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Vol. 30, No. 4 December 1953

The Journal of Experimental Biology

EDITED BY

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Published for The Company of Biologists Limited
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AGENT FOR U.S.A.
ACADEMIC PRESS INC.,
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Price 21s. net *Subscription per volume 63s. net*

THE JOURNAL OF PHYSIOLOGY

NOVEMBER 1953. VOL. 122, NO. 2

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VOL. 30, NO. 4 DECEMBER 1953

OBSERVATIONS ON CERTAIN MECHANICAL PROPERTIES OF THE LIGAMENT OF *PECTEN*

BY E. R. TRUEMAN
Department of Zoology, University College, Hull

(*Received* 19 *September* 1952)

INTRODUCTION

The hinge ligament of most lamellibranchs is a horny structure situated in the dorsal region of the shell, which causes the opening of the valves when the adductor muscles relax. The morphology, development, nature, and certain aspects of the operation of the hinge ligament have recently been described (Trueman, 1949, 1950, 1951) and the basic structure of the ligament and of the mantle secreting it discussed (Owen, Trueman & Yonge, 1953).

Apart from the paper of Marceau (1909) there is little information on the mechanical properties of the ligament. This is remarkable in view of the attention that has been given to the adductor muscles (e.g. Bayliss, Boyland & Ritchie, 1930) and to the swimming habit in the Lamellibranchia (Yonge, 1936) with particular reference to *Pecten*.

The present paper gives a brief description of the ligament of *P. maximus* and discusses experiments to determine certain mechanical properties related to their form.

THE STRUCTURE OF THE LIGAMENT

The ligament of *P. maximus* is situated between the valves dorsally and extends both in front of and behind the umbo. It consists of two main regions, the outer and inner layers (Fig. 1).

The outer layer, uniting the dorsal margins of the valves, which are elongated in *Pecten* to form the auriculae, is laminated, the laminae lying nearly parallel to its ventral surface. In transverse section the layer is approximately rectangular in shape, the width between the valves being much smaller than its thickness. Apart from the laminations this region of the ligament is undifferentiated, and it may be thus considered as a single layer. This layer may represent either the fusion layer or the true outer layer of the ligament (Owen *et al.* 1953). For convenience of reference it is called herein the outer layer, and a full discussion of its interpretation will be published shortly.

The relatively large, dark brown, inner layer of the ligament is attached to the valves in the ligament pits or resilifers. It is pyramidal in shape, with a base that bulges ventrally particularly when the valves are closed. The inner layer may be divided into three parts, a large non-calcareous central region of rubber-like consistency and two lateral calcified regions attaching the former to the valves (Fig. 2). The lateral parts of the inner layer are rather similar to the entire inner layer of the ligament of other lamellibranchs, but the central region appears to be a modification characteristic of the Pectinidae. It consists mainly of a tanned protein complex of a somewhat gelatinous appearance. The outer layer is much more fibrous and is generally similar to the outer layer of the ligament of other lamellibranchs, e.g. *Ostrea* and *Tellina* (Trueman, 1951, 1949).

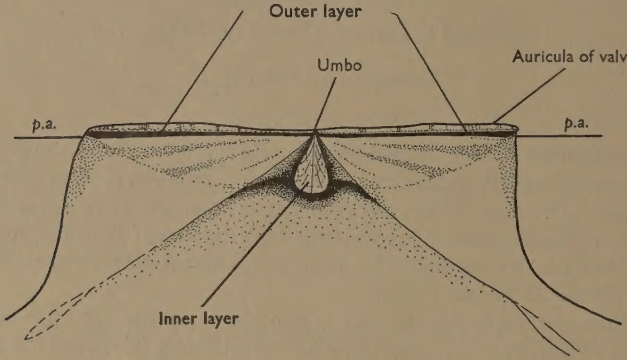

Fig. 1. Interior of the dorsal region of the right valve of *Pecten maximus* with the ligament cut in longitudinal section. The position of the pivotal axis (*p.a.*) is indicated. Approximately natural size.

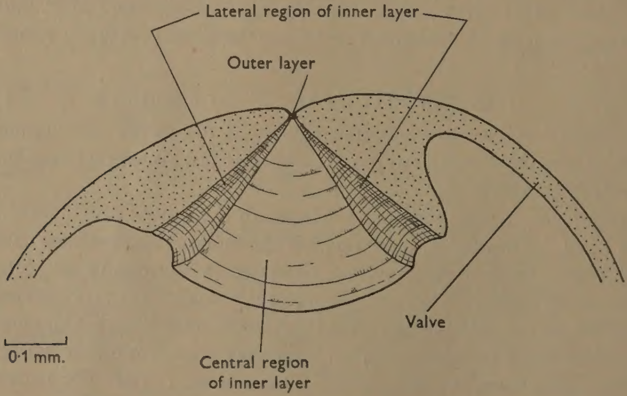

Fig. 2. Diagrammatic transverse section through the ligament of *Chlamys opercularis* showing the outer layer and the central and lateral regions of the inner layer. The outer layer is commonly worn away in this part of the ligament.

It may be noted that the ligament of *Chlamys opercularis* is very similar to that of *Pecten maximus*, and that in some experiments it has been used in place of the latter.

A more complete account of the structure and nature of the ligament of *Pecten* will be published shortly.

MECHANICAL PROPERTIES OF THE LIGAMENT OF *PECTEN*

(1) *The closing moment of the ligament*

The ligament of most lamellibranchs consists of two layers, the outer generally situated above, and the inner layer below the neutral axis of the structure. The former layer is subjected to tensile stress and the inner layer to compression when the valves are closed. The force so derived tends to open the valves and has been measured in terms of a moment about the hinge or pivotal axis of the shell (Fig. 1). In many lamellibranchs the pivotal axis lies in the plane of the neutral axis of the ligament. This force is thus referred to as the opening moment of the ligament, while that required to close the valves may be estimated and referred to as the closing moment of the ligament.

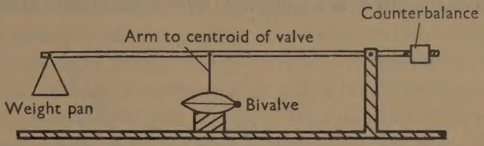

Fig. 3. Diagram of the apparatus used to determine the opening and closing moments of the ligament. A full description is given in the text.

The method used to determine the opening moment of the ligament of the oyster has been briefly described (Trueman, 1951). The equipment consists of a beam, bearing a weight pan and an arm on one side of a pivot, and an adjustable counterbalance weight on the other (Fig. 3). A bivalve shell, from which the tissues have been carefully removed immediately prior to the determination being made, is placed firmly on an adjustable platform with the valves held closed and the counterbalance adjusted so that the arm just rests in a small hole drilled near the centroid of the upper valve. When the valves are released the shell opens and the beam is raised. The weights placed on the pan to close the valves or to hold them in any partly closed position may thus be determined and the effect of their removal observed.

The closing moment of the ligament may be readily determined by means of the equipment described above, measuring the weight required just to close the valves and using the formula

$$\text{closing moment } (M_c) = (2W + v)d \text{ g.mm.},$$

where W = the weight applied (multiplied by 2, since the pan is twice the distance of the arm from the pivot),
v = the weight of the valve, assuming that the point of application of W is close to the centre of gravity of the valve,
d = the distance of the point of application of W from the pivotal axis.

To obtain some basis of comparison for the closing moments of different species it was necessary to take into account the size of the specimen. The closing moment of a number of specimens of the same species, but of different size, was plotted logarithmically against the length of the shell. This showed that the cube of the length gave the most satisfactory relationship, and accordingly the volume of the shell was taken as a convenient measure of size. Thus the ratio closing moment/shell volume (M_c/V g.mm./c.c.) was determined. The results for the ligament of *P. maximus*, *Chlamys opercularis* and a number of characteristic lamellibranchs are given in Table 1.

These results show a fairly wide range of values for this ratio and it is noteworthy that those of *Pecten* and *Chlamys* are similar and are at the lower end of the range. The ligaments of *Mytilus* and *Cyprina* appear to be amongst the most powerful.

(2) *The loading and unloading cycle and the opening moment of the ligament*

If a load-extension curve is drawn for the loading and unloading of an elastic material the form of the curve is a loop, the 'hysteresis loop'. It is possible to draw a similar curve for the ligament (Fig. 4), the load being represented by the weight applied to the valve, expressed in terms of a moment about the pivotal axis of the ligament, and the extension by the angle of gape of the valves. Clearly the state of maximum stress of the ligament is in the closed position of the valves, so that in plotting such a curve it is convenient to show the position of maximum gape

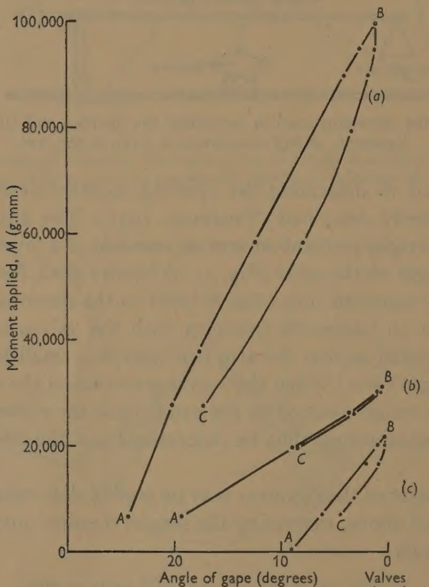

Fig. 4. Graph showing the relation between the load applied to the valve, expressed as a moment 'M' about the pivotal axis, and the angle of gape for the loading (AB) and unloading (BC) of the ligament of (*a*) *Cyprina islandica*, (*b*) *Pecten maximus*, (*c*) *Mytilus edulis*.

(corresponding to minimum stress) on the left of the graph. The loading curve (Fig. 4, *AB*) corresponds with the closure of the shell and the unloading curve to its opening (Fig. 4, *BC*).

The loading curve of the ligament of many species of lamellibranchs (thirty-five species investigated), e.g. *Pecten maximus*, *Chlamys opercularis*, *Cyprina islandica* and *Mytilus edulis*, is a near linear relationship between the force tending to close the valves and their angle of gape. This curve indicates the conditions through which the adductor muscles contract when closing the shell, and shows that the force required is fairly constant per degree of gape over the entire range of gape of the valves.

A consideration of the unloading curve (Fig. 4, *BC*) of the ligament is important, for it is upon the upper part of this curve that the ligament operates in causing the valves to open. When the weight, W, which just closes the shell, is reduced slightly the valves do not immediately open. They commence to gape only when the thrust of the ligament exceeds the pressure on the valves. This thrust of the ligament may be measured as the opening moment (M_o), and it is convenient to determine this and the opening moment per degree of gape of the valves from the unloading curves. The latter are best drawn with the load expressed as the ratio M_o/V in a similar manner to that described for the closing moment. Such curves are shown in Fig. 5 and the results of the determination of the opening moment in Table 1.

Table 1. *Results of the determination of the opening and closing moments of the ligament of a number of typical lamellibranchs*

(The mean closing moment (M_c), the estimated mean opening moment (M_o), and the opening moment per degree of gape (θ) have been expressed in relation to the volume (V) of the shell.)

Species	Number of specimens	$M_c/V \pm$ s.e. (g.mm./c.c.)	M_o/V	$M_o/V/\theta$ (g.mm./c.c./degree)
Mytilacea: *Mytilus edulis*	30	780 ± 48	660	65
Pectinacea: *Pecten maximus*	7	167 ± 16	160	5
Chlamys opercularis	29	148 ± 3·8	142	5
Ostreacea: *Ostrea edulis*	6	425 ± 24	370	40
Cyprinacea: *Cyprina islandica*	2	610	550	25
Cardiacea: *Cardium edule*	35	140 ± 10	114	5
Mactracea: *Lutraria lutraria*	8	285 ± 5·3	265	20
Tellinacea: *Tellina crassa*	29	370 ± 13	320	9

Examination of the hysteresis loops drawn for different ligaments in Figs. 4 and 5 shows that the difference between the loading and unloading curves is at a minimum in the case of *Pecten maximus* and *Chlamys opercularis*. Similarly, from Table 1 it may be observed that these species show least difference between the closing and opening moments. The difference between these moments is some measure of the internal resistance of the structure. It is approximately 4 % of the closing moment in *Pecten*, but values of between 10 and 20 % are commonly encountered in other bivalves. The relatively low internal resistance of the ligaments of *Pecten* and *Chlamys*, and their correspondingly low 'work-loss', suggests that these structures are more efficient for the frequent opening and closing of the valves than are those of the other species examined.

Only in a few lamellibranchs do the valves open to the maximum gape, the ligament normally operating over the top portion of the curves shown in Fig. 5. The experimental loading and unloading of the valves was arranged, in a number of species, so that a curve could be drawn representing loading from a position of maximum gape, partial unloading and the subsequent reloading of the ligament. Such curves are shown in Fig. 6 for the ligaments of *Mya*, *Anodonta* and *Chlamys*, the operation being carried out in a period of 10 min. From this figure it may be observed that the second loading curve is commonly to the right of the initial loading curve. Successive unloading curves however remain relatively constant.

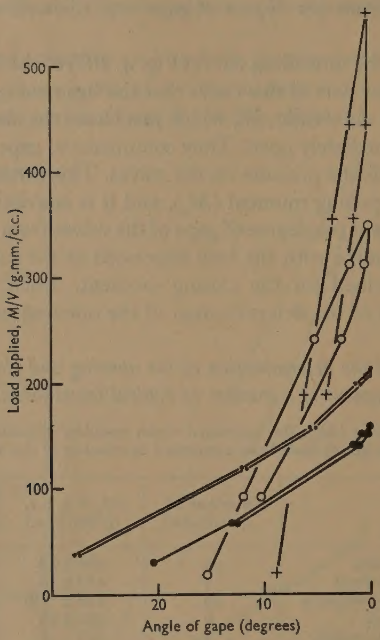

Fig. 5. Graph showing the relation between the load applied to the valve, expressed as a ratio between the moment '*M*' and the shell volume '*V*' (*M*/*V*), and the angle of gape for the following species: +——+, *Mytilus edulis*; O——O, *Lutraria lutraria*; •——•, *Pecten maximus*; ●——●, *Chlamys opercularis*.

Repeated experimental opening and closing of the valves, e.g. 100 times in 1 min. in *Chlamys opercularis*, 100 times in 2 min. in *Pecten maximus* or 160 times in 4 min. in *Anodonta cygnea*, through an angle of gape restricted to about 10 degrees, showed practically no variation in the unloading-loading cycle. From these observations it may be concluded that there is little change in the elastic properties of the ligament of these species due to the repeated opening and closure of the valves over relatively short periods. A comparison of the area enclosed in such loops as these latter (Fig. 6) is possibly a better means of measuring the efficiency of the various ligaments than consideration of the complete loading-unloading cycle. The relatively small loop of the ligament of *Chlamys* is again apparent.

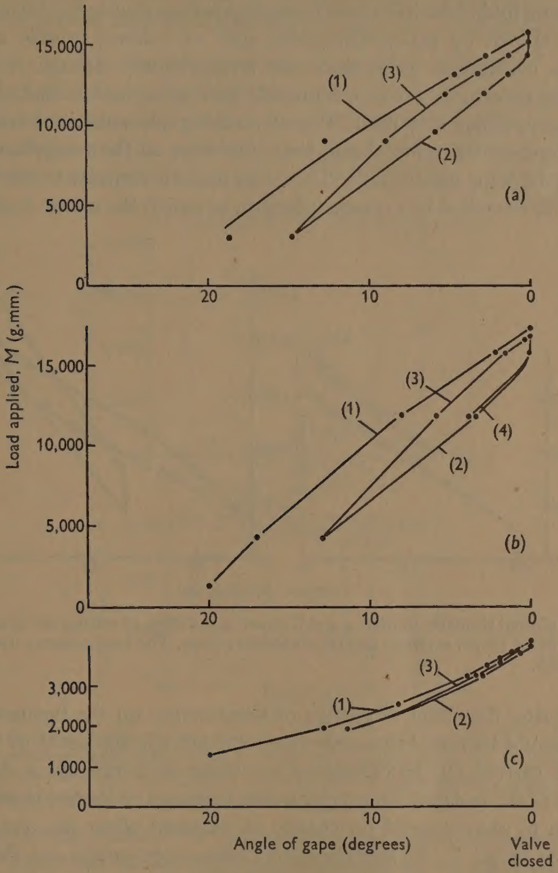

Fig. 6. Graphs showing the effect of loading (1), partial unloading (2), and subsequent reloading (3) and unloading (4) of the ligament of the following species: (*a*) *Mya arenaria*, (*b*) *Anodonta cygnea*, (*c*) *Chlamys opercularis*. The load applied to the valve expressed as a moment (M) is plotted against the angle of gape.

(3) *Thermoelastic properties*

Study of the thermoelastic properties of natural high polymers has been most useful in obtaining insight into the shape and properties of their molecules. The presence of long folded flexible chain molecules is responsible for the high reversible extensibility shown by many substances such as rubber, muscle and collagen. Stretching is considered to produce the straightening of such flexible chains, and when the deforming force is removed, thermal agitation leads the chains to return to a more folded condition. If such a rubber-like substance is stretched and the length kept constant the elastic force increases as the temperature rises and decreases as the temperature falls. This is in marked contrast to the behaviour of a normal solid, stretched to a constant length, in which the elastic force diminishes on warming.

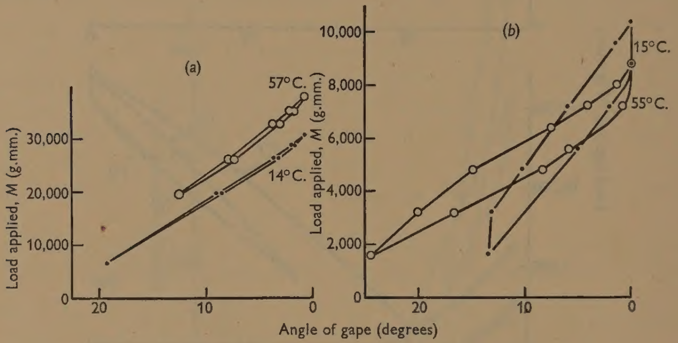

Fig. 7. Graph plotted similarly to Figs. 4 and 6 showing the effect of raising the temperature on the ligaments of (*a*) *Pecten maximus* and (*b*) *Anodonta cygnea*. The temperatures used are indicated on the graph.

In considering the effect of change of temperature on the ligament of lamellibranchs it should be noted that only the outer layer is subjected to tensile stress. Experiments carried out to determine the effect of temperature change on the compression of the isolated inner layer of the ligament of *Pecten*, using the method described on p. 463, showed no change of moment when the temperature was raised from 15 to 50° C. In specimens of *Chlamys opercularis* and *Pecten maximus* the effect of raising the temperature on the loading cycle is to produce an increased moment at all angles of gape (Fig. 7*a*). When the temperature is raised from 15 to 55° C., the ligaments of *Anodonta cygnea* and *Mytilus edulis* show little change at low angles of gape, but at wider angles, i.e. conditions of little stress, the opening moment is increased (Fig. 7*b*). When such specimens are returned to the lower temperature, the loading-unloading cycle generally reverts to normal. Since the temperature change does not appear to affect the inner layer of the ligament the increased moment may be attributed to the contraction of the outer layer as the temperature is raised.

At all stresses normally encountered in the ligaments of *Chlamys* and *Pecten* the temperature coefficient is positive, and the outer layer of the ligament behaves as if composed of long flexible chain molecules. This also occurs at low stresses in the ligaments of *Mytilus* and *Anodonta*. When the valves of the latter are closed the stress is much greater and the temperature change has no positive effect.

(4) *The role of the outer layer of the ligament*

The method used for the determination of the closing moment of the ligament may be utilized in the assessment of the function of different regions of the ligament. Parts of the outer layer of the ligament of *Pecten* or *Chlamys* may be easily removed, because of its location along the auriculae of the valves (Fig. 1), and the effect on the closing moment of the ligament observed. The outer layer of the ligament of *Pecten maximus* was removed in some specimens, first from the anterior and posterior margins of the auriculae, successively cutting away more central portions (Fig. 8*a*), and in other specimens first from the region close to the inner layer, then progressing centrifugally (Fig. 8*b*). Very little, if any, functional outer layer was left above the inner layer of the ligament in the latter procedure.

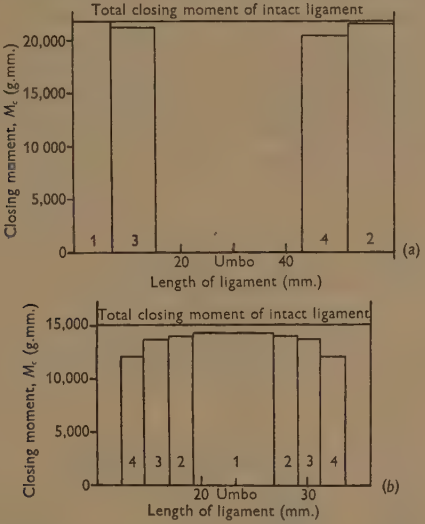

Fig. 8. Graphs showing the effect of removing the outer layer of the ligament on the closing moment (M_c). The columns numbered 1 to 4 indicate the successive parts of the ligament removed (*a*) centripetally, and (*b*) centrifugally. The height of each represents the closing moment of the ligament with that portion of the ligament removed.

It may be observed from Fig. 8*a* that the lateral parts of the outer layer can be removed with little effect upon the total closing moment of the ligament, but that the amount that can be cut away is limited. Below this limit the stress applied to the reduced outer layer, when the valves are closed, causes the tearing of this layer and the fracturing of the inner layer. Removal of the central part of the outer layer

(Fig. 8*b*) shows that a considerable portion may be taken away with little effect, e.g. with only about one-third of the outer layer functional the closing moment is not greatly effected. Complete removal of the outer layer either from in front of or from behind the umbo leads to the fracturing of the inner layer. This is presumably caused by the then asymmetry of the arrangement of the ligament.

These experiments show that considerable parts of the outer layer of the ligament of *Pecten* may be removed without any great effect on the strength of the ligament and possibly indicate that no part of the outer layer is normally subjected to extreme stress. This may be related to its considerable relative length. This view is supported by the small width of the outer layer between the valves (Fig. 2), by its faint birefringence when seen in transverse section and by the similarity of the properties of the isolated inner layer to those of the inner layer of the intact ligament, as discussed in the following section. The outer layer produces a long fairly rigid hinge line near the pivotal axis of the opening of the valves, and is probably of importance in preventing any rotation of the valves on each other, particularly when they are gaping. The association of a single adductor muscle with a relatively long, straight and symmetrical hinge should be noted.

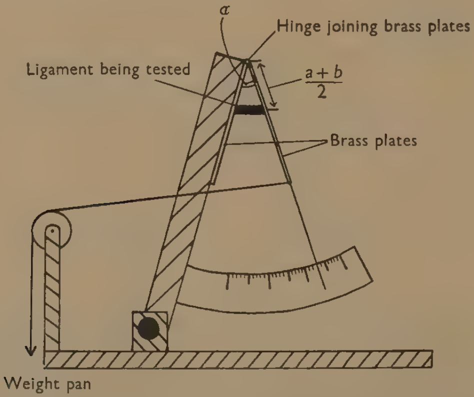

Fig. 9. Diagram of the apparatus used to investigate the compression of the isolated inner layer of the ligament. For further explanation see the text.

(5) *The properties of the isolated inner layer*

The results of the experiments described above indicate the importance of the inner layer of the ligament of *Pecten*. To enable the properties of this layer of the ligament to be investigated under conditions as similar as possible to those found in life, but without the complication of the presence of the outer layer, the following equipment was used.

Two brass plates were firmly hinged together, one attached to a wooden arm and the other hanging free with a pointer extending to a scale on which the angle (α, Fig. 9) could be read off. Pieces of ligament cut from the inner layer were placed between the plates, orientated the same way as between the valves, and subjected to compression by means of weights attached to the end of the free plate using the pulley wheel shown in Fig. 9. The main arm was adjustable, allowing the free plate to be moved into a vertical position so that its weight need not affect the calculations.

Using this equipment portions of the inner layer of the ligament can be subjected to compression and the angular change recorded. From these figures the modulus of elasticity under compression can be determined and stress-strain (or load-compression) curves may be drawn.

Young's modulus of elasticity in compression may be determined from the formula

$$E=\frac{2M\alpha}{\theta s(a+b)},$$

where E = the modulus of elasticity in compression,

M = the force applied, estimated as a moment about the hinge,

$\frac{a+b}{2}$ = the mean distance from the hinge to the material tested,

s = the cross-sectional area of the material used,

α = initial angle subtended by the arms (in radians),

θ = change in angle subtended by the arms (in radians).

Where possible these symbols are indicated in Fig. 9. The stress-strain curves for the material tested may be drawn, plotting the forces applied per unit cross-sectional area (g./mm.^{2}) against the percentage compression estimated from the angular change.

This procedure was applied to portions of the inner layer of the ligament of *Pecten maximus*, *Ostrea edulis* and *Lutraria lutraria*, and the results are given in Table 2 and Fig. 10.

The mean modulus of elasticity in compression of the inner layer of the ligament of *Pecten* is 320 g./mm.^{2}. This figure is much lower than the values obtained for *Ostrea* (2170 g./mm.^{2}) or *Lutraria* (2240 g./mm.^{2}) and indicates that the inner layer of the ligament of *Pecten* is much less resistant to forces closing the valves than that of the latter two bivalves. The variations in the results obtained for *Ostrea* and to a lesser extent those of *Lutraria* are probably due to the small size of the portions of the ligament obtainable, which consequently had to be placed as near the hinge as possible.

The stress-strain curves (Fig. 10) show that the load necessary to produce 20% compression of the inner layer of the ligament of *Pecten* is approximately one-fifth of that required similarly to compress that of *Ostrea*. The percentage compression undergone by the inner layer of the ligament *in situ* between the valves when the shell closes is about 45% in *Pecten*, 30% in *Ostrea* and 40% in *Lutraria*. Stress-strain curves have also been drawn for the isolated inner layer when subjected to these amounts of compression, with similar results.

The curves shown in Fig. 10 are in the form of the hysteresis loop, and the area of this for *Pecten* is much smaller than that of the other species. This corresponds to the observations on the complete ligament. This, together with the conclusions reached regarding the role of the outer layer of the ligament of *Pecten*, emphasizes the importance of the inner layer of this type of ligament in the determination of its characteristic properties.

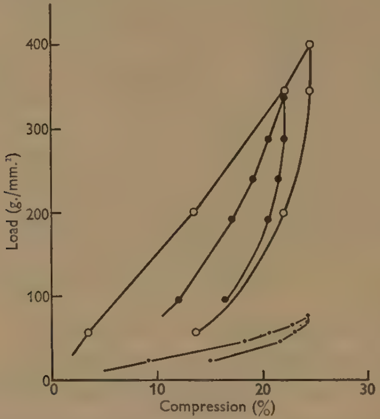

Fig. 10. Stress-strain curves for the inner layer of the ligaments of *Ostrea edulis* (○——○), *Lutraria lutraria* (●——●) and *Pecten maximus* (•——•) when tested in the equipment shown in Fig. 9. The stress is represented by the load applied per mm.² of cross-sectional area under test and the strain by the percentage compression estimated from the angular change.

Table 2. *Results of the determination of Young's modulus of elasticity* (E) *in compression of the inner layer of the ligament of certain lamellibranchs*

Species	Number of specimens	$E \pm$ S.E. (g./mm.²)
Pecten maximus	8	320 ± 16
Ostrea edulis	5	2170 ± 395
Lutraria lutraria	6	2240 ± 92

DISCUSSION

While the ligament of *Pecten maximus* is structurally and functionally comparable to that of other lamellibranchs, it shows two important differences: first the elongated fusion or outer layer, and secondly the presence of an unusual central region of the inner layer. These modifications are found in other genera of the Pectinidae, e.g. *Chlamys*, *Amussium* and *Hinnites*.

The function of different parts of the ligament of *Pecten* has been referred to above, the outer layer being subjected to tensile stress and the inner to compression when the valves are closed. The outer layer probably behaves as a fairly rigid hinge structure, and the peculiar characteristics of this ligament may be attributed very largely to the central region of the inner layer. The relatively small area enclosed in the hysteresis loops drawn for the ligaments of *Pecten* and *Chlamys* (Figs. 4 and 5), the correspondingly low work-loss and the similarity of the opening and closing moments (Table 1) are features of this type of ligament. Furthermore, it should be noted that the ligament of *Pecten* opens the valves to a wide angle (approximately 30 degrees) as compared with 10 degrees for *Mytilus edulis* or *Ostrea edulis*. Accordingly, the opening moment per degree of gape is relatively low for *Pecten* (Table 1). Another characteristic of the ligament of *Pecten* is the rapidity of change of angle of gape with variation of the load on the valve. The change is effected almost instantaneously in *Pecten*, but in bivalves such as *Mytilus* or *Anodonta* there is both an instantaneous effect and a subsequent movement lasting over several minutes. Allowance was made for this when measuring the closing moment of such lamellibranchs. Thus the inertia of the ligament of *Pecten* appears to be lower than that of other ligaments. These properties may be largely attributed to the central region of the inner layer of the ligament.

It is significant that this region is markedly different in composition from the inner layer of the ligament of other lamellibranchs. The lack of calcification of the inner layer, except for the lateral parts, is the main difference between this layer in the ligament of *Pecten* and that of other lamellibranchs, e.g. *Lutraria* or *Ostrea*. It may be considered that the effect of calcification is to increase the resistance of the inner layer to compression. This would account for the higher modulus of elasticity in compression of the inner layer of the ligament of *Ostrea* and *Lutraria* (Table 2). It is probable also that calcification increases the internal resistance of the material of the ligament, so that the relative efficiency is decreased at the same time as the modulus is increased. It may be noted that the increase in the modulus of the inner layer corresponds with the rather more substantial outer layer of the ligament of *Lutraria* and *Ostrea*, and a greater opening moment than that of *Pecten*.

It is noteworthy that the ligament of *Pecten maximus*, which is representative of the small number of lamellibranchs that are free-swimming, is a more efficient structure for the opening and closing of the valves than that of *Ostrea* or *Lutraria*.

The evolution of the swimming habit has been discussed by Yonge (1936), who points out that with the exception of the functional asymmetry of the statocyst in *Pecten*, the adaptations present in swimming Lamellibranchia are no more than further developments of those originally acquired for the efficient cleansing of the mantle cavity. The opening and closing of the shell of *Ostrea* for the purpose of cleansing the mantle cavity is fairly frequent, especially in water where the rate of sedimentation is high (Nelson, 1921). But the ligament of *Pecten* has certain characteristics, such as the wide angle of gape, and its relatively high efficiency, which would seem to be advantageous for swimming. It is possible that they were initially related to the cleansing of the mantle cavity but they would certainly be an asset in the adoption of the swimming habit.

SUMMARY

The ligament of *Pecten maximus* consists of two layers, the outer extending along the dorsal margin of the valves, and the inner situated between the valves in the region of the umbo. The former appears fibrous and is somewhat similar to the outer layer of other ligaments. The inner layer consists of three parts, a large central non-calcified structure and two lateral calcified regions attaching the former to the valves. The central region is characteristic of the ligament of *P. maximus* and associated species, this layer being uniformly calcified in the ligament of most bivalves.

When the valves are closed the outer layer is subjected to tensile stress and the inner to compression, and the force so derived tends to open the valves. This force and that required just to close the shell are expressed as the opening and closing moments of the ligament respectively, and these have been determined for various lamellibranchs.

The exact conditions of the opening and closing of the valves have been observed by drawing the stress-strain curves for the intact ligament, plotting the applied moment against the angle of gape. The loading and unloading curves so produced describe a hysteresis loop. The area enclosed in that of *Pecten* (or *Chlamys*) is markedly less than that of other lamellibranchs and indicates the greater efficiency of the ligament of the former.

The mechanical properties of the isolated central region of the inner layer of the ligament of *Pecten* were investigated and showed similar properties to those of the intact ligament. The modulus of elasticity in compression of this region of the ligament of *Pecten* is approximately one-seventh of that of the inner layer of *Ostrea* or *Lutraria*, which are calcified structures. The ligament of the latter two bivalves has a greater opening moment but lower efficiency than that of *Pecten*. The characteristics of the ligament of *Pecten* are probably due to the central non-calcified region of the inner layer. The significance of these properties in regard to the swimming habit of *Pecten* is briefly discussed.

The author is indebted to the Challenger Society and to the research fund of the University College of Hull for grants to defray expenses incurred while working at the laboratories at Millport and Plymouth respectively, and wishes to thank the staff of those institutions for their assistance in providing the material used in this work. The author is most grateful to Prof. J. F. Baker, O.B.E., for suggesting the equipment shown in Fig. 3, to Mr J. A. Shepperd for advice on the mechanical problems, and to Prof. P. G. 'Espinasse and Prof. C. M. Yonge, F.R.S., for much helpful criticism.

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CENTRAL NERVOUS FUNCTION AND CHANGES IN BRAIN METABOLITE CONCENTRATION

III. GLYCOGEN IN THE NORMAL BEHAVIOUR OF MICE

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(*Received* 18 *September* 1952)

INTRODUCTION

As reported earlier (Chance & Yaxley, 1950), glycogen increments in the mouse brain are specifically related to the occurrence of convulsive discharge from the central nervous system. Also in that publication it was reported that the convulsion precipitated by shaking the H.C.* strain of mice by the tail was accompanied by glycogen increments in different parts of the brain. Subsequently, it was observed that the H.C. convulsion is identical with the posture adopted by normal mice during a fall (Chance, 1953). It was therefore considered worth while to investigate the possibility that some types of normal mouse behaviour might be accompanied by brain glycogen increments. In this respect the investigation widened the scope of the earlier work which was undertaken to define precisely the conditions in which changes in the concentration of brain metabolites take place in the mouse brain.

METHODS AND RESULTS

Different parts of the brain were examined since the earlier investigations had shown that the extent of the glycogen increment varies from one part of the brain to another, depending on the nature of the precipitating stimulus (Chance, 1951).

Glycogen estimations were made by the method described in the earlier publications (Chance & Yaxley, 1950). The description and identity of the different anatomical parts of the brain, and the values for the normal glycogen concentration of these different parts, were also published in that paper.†

Male mice weighing 20–25 gms. of a single strain were used throughout.

The methods used for inducing the different types of behaviour in mice and the results obtained are described below.

Running

The mice were placed on a horizontal rotating disk, with a rough surface, diameter 12 in. Above the disk two fixed vertical wooden barriers were mounted. They met above the centre of the disk and lay along radii diverging at an angle of

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* Animals of this strain adopt a characteristic posture when lifted by the tail and momentarily lowered sharply. The posture may be maintained for 5 sec.

† A decimal error has occurred in columns 2 and 3 of the table of normal values published in the paper by Chance & Yaxley (1950). The figures should be multiplied by ten to give the correct values.

60°. The gap between their ends, $7\frac{1}{2}$ in. from the centre, was closed by a tangential barrier. The barriers thus confined the mouse to the periphery of the rotating disk.

The disk was then rotated, slowly at first, and finally at a constant rate which, at the maximum, forced the mouse to run at a speed of 8·8 yd./min. At higher speeds the mouse fell off the edge. The figures in Table 1 show that the glycogen concentration remained unchanged throughout the brain, after it had run 120 yd. in 13 min.

Table 1. *Running*

Description of events	Percentage change in glycogen content		
	Cortex	Mid-brain	Medulla
Six mice each forced to run 120 yd. in 13 min. (i.e. 8·8 yd./min.)	−4·2	−0·7	0
	+1·3	0	+1·8
	+6·7	−0·3	0
	−1·0	−1·5	+1·6
	+1·8	0	0
	+4·0	+0·7	0
Mean change	+1·4	−0·3	+0·6

Swimming

Mice swim as a reflex to immersion in water, and provided there is no point at which they can climb out, the mice 'dog paddle' continuously. In this experiment the mice were allowed to swim round an aquarium tank with vertical sides for 4 min. Earlier observations had shown that exhaustion sets in after about 6 min. No change in brain glycogen occurred (see Table 2).

Table 2. *Swimming*

Description of events	Percentage change in glycogen content		
	Cortex	Mid-brain	Medulla
Six mice each swam continuously for 4 min. in water at 34° C.	0	−3·4	0
	−3·1	−2·8	+1·9
	+5·3	−1·7	+1·1
	−0·4	−1·7	+0·8
	+3·5	−0·8	0
	0	−0·8	−0·3
Mean change	+0·9	−1·9	+0·6

Jumping

When a mouse is kept in a glass beaker it will often jump vertically up on to the edge. This behaviour can be induced by the application of an electrical stimulus to the feet of the mouse. For this purpose, a square perspex container with sides 4 in. high and with a metal grid floor was constructed with bars $\frac{1}{10}$ in. apart. Alternate bars were connected together along one side and reached to within $\frac{1}{10}$ in. of the opposite side. In this way alternate bars formed the opposite poles of an electric circuit which was connected through a tapping key to a 'variac' by means of which the voltage in the circuit could be altered. The feet of the mouse made contact with more than one bar at a time, and thus completed the circuit. By depressing the tapping key, and so closing the circuit, a short series of electrical shocks was applied to the feet, and, at a sufficiently high voltage, induced the mouse to jump up to the edge of the container. To induce a jump down the box was reversed on the bench so that the grid was at the top. The figures in Table 3 show that a glycogen increment occurred in the cortex of all the mice which jumped *up*, and in five out of the six mice which jumped *down*. No change took place in the mid-brain or medulla. A control test with a just subthreshold electrical stimulus produced a slight rise in the cortex by an amount which is not statistically significant from the cortical values of the twenty-two normal mice, the mean figure for which was published earlier (Chance & Yaxley, 1950). This indicates that the cortical increment is a result of the jumps.

Table 3. *Jumping*

Description of events	Percentage change in glycogen content		
	Cortex	Mid-brain	Medulla
Six mice each stimulated by 60 V. 50-cycle current in platform grid to jump *up* a height of 4 in. On three successive occasions	+6·7	0	+1·8
	+9·3	−0·7	+1·8
	+10·3	−0·4	0
	+4·0	0	+1·8
	+9·3	+0·4	+1·8
	+9·3	+0·8	0
Mean change	+8·1	+0·1	+1·2
Six mice each stimulated by 60 V. 50-cycle current in platform grid to jump *down* a height of 4 in. On three successive occasions	+6·7	+0·7	0
	+6·7	0	+1·8
	0	−0·4	+3·0
	+9·3	+1·8	+0·4
	+6·7	−0·4	0
	+4·0	−0·7	+1·8
Mean change	+5·6	−0·1	+1·2
Control observations; six mice stimulated on three successive occasions with 50 V. 50-cycle current. Three mice did not jump *up*	+2·7	+1·2	0
	+4·0	0	−0·8
	0	+1·7	−0·8
	+2·7	—	—
	0	—	—
	+1·3	—	—
Mean change	+1·7	+1·0	−0·5

Falling

The falling mouse adopts a characteristic posture which has been described by Chance (1953). It appears to be a posture which, on landing, serves to prevent damage by concussion to internal organs. The final posture is the result of two separate reactions on the part of the falling animal. The first—the falling reaction—elicits the characteristic posture of the ears, jaws, head, body and limbs, and the second—the righting reaction—by which the body is rotated on its axis, brings the feet below the body. This only takes place if the body is tilted from the normal position at the start of a fall. The falling reaction can be elicited alone by withdrawing support from an animal, or it can be combined with the righting reaction by tipping the support, and so allowing the animal to slide off sideways. The effect of each of these possibilities has been examined on the brain glycogen, after allowing the animal to fall in the same way on three successive occasions, at intervals of less than ½ min., from a height of 14 ft. on to a folded blanket. The duration of each fall was 0·7 sec. approximately. The values of the glycogen in the different parts of the brain, listed in Table 4, show that increments occur in all parts and, with the exception of the mid-brain, are the same for the two procedures. The mid-brain increment is low following loss of support alone, and rises to approximately the same value as that in the medulla on the addition of the righting reaction. It is concluded that the mid-brain increment is a reflexion of the activity in the brain due to the righting reaction; and that, if the impact is assumed to play no part, the increments in the cortex and medulla result from brain activity associated with the falling posture. Comparison of the figures following loss of support alone with those reported in figure 9 of the earlier publication (Chance & Yaxley, 1950) for H.C. mice held by the tail and shaken show that the pattern is the same. This supports the conclusion that the cortical and medullary increments are the result of the falling posture and are not due to the stimulus of impact. Added support for this is obtained from the glycogen increments on falling, which are higher for H.C.

Table 4. *Falling mice*

Description of events	Percentage change in glycogen content				
	Cortex	Mid-brain	Medulla	Cerebellum	
				Vermis	Lateral lobes
Six mice each allowed to fall from a horizontal position, a distance of 14 ft. on three successive occasions (=loss of support)	+60·0	+4·0	+22·0	+13·0	+21·0
	+44·0	+8·0	+33·0	+20·0	+27·4
	+52·0	+2·9	+29·5	+20·0	+29·5
	+60·0	+10·2	+33·4	+22·4	+27·4
	+68·0	+2·9	+27·8	+20·0	+21·0
	+57·3	+8·0	+29·5	+15·0	+29·5
Mean change	+56·9	+6·0	+29·2	+18·4	+25·9
Six mice each allowed to fall from a laterally tilted horizontal position, a distance of 14 ft. on three successive occasions (=loss of support+righting reaction)	+58·5	+14·1	+18·5	+15·0	+29·5
	+57·0	+50·0	+26·0	+17·0	+22·5
	+54·5	+25·5	+29·5	+20·0	+27·0
	+57·0	+20·0	+25·0	+20·0	+20·6
	+60·0	+21·1	+25·0	+22·4	+24·0
	+57·6	+20·5	+27·2	+15·0	+12·6
Mean change	+57·4	+25·2	+25·2	+18·2	+22·7

Table 5. *H.C. mice falling*

Description of events	Percentage change in glycogen content				
	Cortex	Mid-brain	Medulla	Cerebellum	
				Vermis	Lateral lobes
Loss of support (as in Table 4)	74	14	48	23	23
	83	24	43	21	25
	81	19	57	—	—
Mean change	79	19	49	22	24
Loss of support+righting reaction (as in Table 4)	78	46	48	24	21
	82	51	43	24	24
	82	51	43	—	—
Mean change	81	49	45	24	22

mice (see Table 5) than for normals (see Table 4). This difference shows that the increased responsiveness to loss of support is reflected in the amount of the glycogen increment as well as in the ease with which the falling posture is elicited.

Fighting

This behaviour is shown by young adult male mice of most strains after they have been isolated for a period lasting between 3 and 6 weeks. When they are removed from their isolation boxes and are placed together into another box they start to threaten each other by rattling the tail, and will often fight for a period which rarely exceeds half an hour. The fight is terminated by one mouse offering the ‘defensive posture’ (Chance, 1947) to the approaches of the aggressor, and on all subsequent encounters either submitting to bites, adopting the ‘defensive posture’ or running away. In these circumstances, the aggressor gradually lowers the intensity of its attacks till tail-rattling replaces attacks, and finally it retires to another part of the box. Careful observation of many such encounters shows that, in most instances the mouse which finally adopts all three submissive gestures has adopted the ‘defensive posture’ early in the fight, but that this has not appeared (except occasionally once or twice) in the repertoire of the mouse which is finally dominant. The fight appears to persist as long as the behaviour of the ultimately submissive mouse contains retaliatory attacks in response to the aggressive advances of the more dominant animal. In respect of its role as aggressor or attacked, the behaviour of any mouse is usually consistent over the greater part of the fight.

Table 6. *Fighting*

Period of fighting (min.)	Percentage change in glycogen content		
	Cortex	Mid-brain	Medulla
	Submissive mice		
10	+1·3	−2·8	0
14	−1·3	+2·8	+0·7
19	−2·7	−2·8	+1·9
22	0	+2·0	0
26	+1·3	−3·0	+1·9
33	−1·3	−2·8	+2·0
Mean change	−0·4	−1·1	+1·1
	Dominant mice		
20	+24·5	+2·8	+1·8
25	+33·0	0	+3·7
28	+ 6·7	+2·8	+1·8
30	+13·7	−0·8	+1·8
35	+13·0	−0·4	+1·8
36	+33·0	−0·8	+3·2
Mean change	+20·6	+0·5	+2·3

The fight was considered to be over 5 or 10 min. after the consistent adoption of the defensive posture’ by the submissive mouse had terminated attacks by the aggressor.

The brain glycogen was estimated in six submissive and six dominant mice following twelve typical fights of the type described. The results shown in Table 6 reveal that increments of cortical glycogen occurred in all dominant animals. The extent of the rise in the medulla, although constant, is not statistically significant ($P=0{\cdot}1$) when comparison is made with the mean medullary value for the twenty-two normal mice already published (Chance & Yaxley, 1950).

A detailed analysis of the fighting behaviour of mice is under way, but as yet it has not been possible to make any suggestions as to the components of the behaviour which are responsible for the glycogen increment in the brain of the dominant mouse.

Sleeping

Mice can be induced to sleep in quiet surroundings if placed under a bright light. Sleep is recognized by a deep regular breathing at about 120/min., and the mouse is usually lying on its side and curled up. When touched on the back once sufficiently hard to straighten the spine a small amount, it does not open its eyes or move any part of the body. It may be roused on a second touch. This test distinguishes sleep from resting; in the latter condition the eyes are often closed but the respiration is less regular. A single touch on the back will cause a resting mouse to open its eyes and raise its head. It may also move away. As shown in Table 7 the brain glycogen content was not altered by sleep which lasted 15 min.

Table 7. *Sleeping*

Description of events	Percentage in glycogen content		
	Cortex	Mid-brain	Medulla
Six solitary mice in cages placed 22 in. under 100 W. lamp in quiet room until asleep. Left asleep 15–20 min.	+1·2	0	0
	0	+0·7	−0·8
	−2·2	0	+0·8
	+1·2	+0·7	+0·8
	−2·2	+0·7	−0·8
	−2·2	0	−0·8
Mean change	−0·7	+0·4	−0·1

SUMMARY

1. The changes in the glycogen content of different parts of the brain have been studied in mice in relation to the behaviour of these animals.

2. Increases in glycogen content have been demonstrated after a jump, a fall, after the righting reaction accompanying a fall, and after aggressive behaviour has been shown in a fight.

3. No increase has been demonstrated during sleep, after running or walking, or after defensive behaviour in a fight.

4. It was shown earlier that external stimulation must reach convulsive intensity to produce an increase in brain glycogen, and it is suggested that the particular forms of normal behaviour associated with increase in brain glycogen involve a 'convulsive' type of brain activity.

'Convulsive' in this context may mean either a disturbance of the metabolism in some or all of the brain cells, or alternatively the mobilization of cell populations for simultaneous, and probably rapid, discharge. At present there is no evidence to suggest whether either or both of these possibilities are involved.

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OXYGEN CONSUMPTION AND CELL SIZE. A COMPARISON OF THE RATE OF OXYGEN CONSUMPTION OF DIPLOID AND TRIPLOID PREPUPAE OF *DROSOPHILA MELANOGASTER* MEIGEN

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(*Received* 6 *December* 1952)

INTRODUCTION

The generalization that metabolic rate, per unit of weight, decreases with the size of the animal is certainly valid for birds and mammals; it seems fairly clear that it is also true of poikilotherms (see Weymouth, Crismon, Hall, Belding & Field, 1944; Zeuthen, 1947; Hemmingsen, 1950; and Ellenby, 1951). Apparently the rate of metabolism usually varies as the 0·75 power of the body weight (Kleiber, 1947); the difference of this value from 2/3 has led to the conclusion that metabolic rate is not proportional to the surface-area, and that, in fact, the surface-law is not valid. There are cases, however (Ellenby, 1945*b*, 1951), where it has been shown that, although metabolic rate is not proportional to a 2/3 power, it nevertheless is proportional to the surface area. Now, while there is general agreement about the variation in metabolic rate with increasing body size, the reason for this variation remains obscure. On many occasions it has been suggested that the extent of the surface area of the cells was the causal factor; but the fallacy of this view has been pointed out just as frequently. Rubner (1913), for example, by estimating the surface of the cells showed that such a hypothesis was impossible. Clearly such a factor can only be significant if cells increase in size, but not in number, with increasing body size; this, in general, is not so. But as I have already pointed out (Ellenby, 1945*a*), there are cases in insects in which it is known that all size increase is due to increase in cell size (Trager, 1935; Abercrombie, 1936) and other cases in which some of the increase is due to increase in cell size (Trager, 1935). Of particular relevance is the work of Alpatov (1930), who showed that the size of the cells in *Drosophila melanogaster* could be influenced by rearing the animals on different diets. It was therefore of interest to discover whether the differences in rate of oxygen consumption shown to exist between *Drosophila* prepupae of different sizes (Ellenby, 1938, 1945*a*) could be due to differences in cell size; a line giving triploids provided ideal material for such a study, for, as is well known (Boveri, 1905) nuclear size generally corresponds to chromosome content, and nuclear size, in turn, is related to cell size.

Gowen (1930*a*, *b*) examined the duration of life and the carbon dioxide production of male, female, triploid females, and sex intergrade imagines of *Drosophila*. On the whole the results were in agreement with Rubner's view (1908) that the total energy transformed during the whole life of an animal is an approximate constant. He found no direct correlation between cell size and metabolic rate of the different classes of flies examined. But this work is unsatisfactory in a number of ways: (1) The carbon dioxide production of a number of flies was measured while they were free to move in a stoppered vial; differences of activity of the different classes examined would therefore affect the results. (2) Allowance was not made for the different body sizes of the various types of animals examined and results were calculated per unit of body weight. Body size influences the rate of oxygen consumption of prepupae of *D. melanogaster* (Ellenby, 1937, 1945 *a*), and, possibly, the rate for imagines. On the other hand, the size of an animal may vary independently of the size of its cells; for example a triploid animal with large cells may be the same size as a diploid with smaller cells (Fankhauser, 1945; Beatty & Fischberg, 1951). It is therefore necessary to consider the size of the animals used, as well as the size of their cells. The techniques developed for the study of prepupal oxygen consumption in relation to surface area suggested that examination of the problem would be of interest.

MATERIALS AND METHODS

The progeny of a triploid female mated to a normal diploid male consist of eight different types, due to variations in the ratio between the number of *X*-chromosomes and the number of autosomes present in the cells of a given individual (Bridges, 1921, 1922). Externally, they can only be identified in the imaginal stage: if one wishes to compare the prepupal oxygen consumption of certain of these different forms it is therefore necessary to work with individual prepupae of unknown type rather than with groups, and to identify their type subsequently. The present investigation was restricted to an examination of triploid and diploid females and diploid males, that is, to the forms with 'balanced' chromosomes and very largely to a comparison of the first two forms. The main interest was to compare individuals as similar as possible in all but cell size, as there is good reason for believing that the other categories with 'unbalanced' chromosomes differ in other ways too. The triploid females which it was most desired to examine make up only 3·9% of the progeny (Bridges & Anderson, 1925; Dobzhansky, 1929), but, fortunately, to discover them is not quite as difficult as it sounds, for Dobzhansky (1930) has shown that there are sharp differences in the time of development between the forms with 'balanced' chromosomes and the forms with 'unbalanced' chromosomes. At 27° C., the mean developmental period for diploid females is 199·24 hr., for males it is 205·20 hr., and for triploid females it is 203·83 hr.; on the other hand, the other forms have times ranging from 255 to 272 hr. These times, of course, are for the whole developmental period; but, nevertheless, there seemed little doubt that similar differences would exist in the length of the larval period so that it would be possible to reduce the size of the population which had to be examined for triploid females.

The stock was the same as that used by Dr Sarah Pipkin in her work on triploids (1942); it was homozygous for the three recessive *X*-chromosome mutants *yellow*$_2$, *vermilion*, *forked*, and, at the time the experiments were carried out, had been inbred for about a year. It was maintained at 25° C. in half-pint milk bottles on the maize-meal-agar-molasses medium in use in the laboratory at University College, London.

Breeding techniques were essentially similar to those already described (Ellenby, 1938, 1945*a*). Triploid females, already mated for 4 days, were allowed to lay eggs for 4 hr. in culture bottles yeasted the previous night; they were then transferred to sets of other bottles in turn. As before, it was found possible to obtain prepupae differing greatly in size by varying the number of egg-laying females per bottle.

As the usual paper towelling was not added the larvae crawled up the sides of the bottle to pupate. For reasons already given, it was important to collect prepupae for the respiration experiments as soon as they began to form. As soon as this began to happen all prepupae already formed were removed from the walls of a set of bottles, and the bottles were set aside in the incubator for an hour; prepupae removed at the end of this time were used in the respiration experiments. They were washed, adhering food removed, and their surfaces were sterilized by rinsing with alcohol. Animals showing the slightest movement during these manipulations were rejected. At this stage it was also possible to reject undesired males, their testes being visible through the body wall (Kerkis, 1931) since the prime interest was the comparison of diploid females with triploid females. The remaining prepupae were placed in the respirometers which were then transferred to a water-bath. The respirometers and water-bath were maintained in a constant temperature room so that, despite the large volume of air in the apparatus, an equilibration period was unnecessary; nevertheless, a 15 min. period was allowed so that the first reading was taken after about 1 hr. from the end of the period of collection. Subsequent readings were taken, generally at intervals of an hour, until the experiment had lasted 5 hr.

The constant temperature room had been subjected to prolonged test and had shown itself apparently capable of maintaining very accurate control. Unfortunately, during the course of the experiments the ambient temperature went up considerably and the room proved to be less satisfactory than had at first appeared. Throughout the course of the experiments the mean temperature was $26{\cdot}0 \pm 0{\cdot}5$: however, on any particular day, the temperature of the water-bath varied by less than $\pm 0{\cdot}1^\circ$ C. As the oxygen consumption of a number of prepupae of different sorts was measured simultaneously on any particular day, the fact that the temperature control was less satisfactory than desirable has the effect of increasing the variation but not the validity of any comparison between the different sorts.

As it is only possible to identify triploids in the imaginal stage it was necessary to measure the oxygen consumption of individual prepupae whose constitution could be determined subsequently. Respirometers of a modified Gerard-Hartline type were used (Gerard & Hartline, 1934); these have already been fully described (Ellenby, 1946). Each consists essentially, of two vessels, separated by a drop of manometric fluid; but one vessel, a capillary tube holding the animal and some KOH, is contained within the other, a very large tube, the manometric fluid separating the two. The system is a differential one, but as the volumes of the two tubes are so different, the movement of the manometric fluid is equal to the actual volume change in the capillary due to the oxygen consumption of the animal. In fact, each large tube contained two of the capillaries, specially adapted for the particular task, and six of the large tubes were mounted so that they could be rotated about a horizontal axis. The oxygen consumption of twelve prepupae could thus be measured simultaneously. Movement of the manometric fluid was measured with a traversing microscope reading from lines etched on the capillaries. 1 cm. corresponded to between 2 and 3 cu.mm. depending on the particular apparatus.

The prepupae were weighed on a Fabergé torsion balance (Fabergé, 1938) at the end of each experiment. Each was then gummed to a microscope slide and kept in a vial with moist filter-paper so that the imago could be examined on emergence. Puparial surface area and cell size were both estimated by methods already described. For the former (Ellenby, 1945*a*) the puparia left behind on the slides after emergence were used: each was flattened between two microscope slides and the outlined areas of the projected images measured with a planimeter. As triploids can only be identified in the imaginal stage it is impossible to determine their cell size in the prepupal stage. Cell size was therefore conveniently estimated by measuring the size of the epidermal cells of the wing. It was felt that, as a first step, the assumption that the other cells would show the same relationship was justified, in view of the supporting evidence from other animals (Fankhauser, 1945). Accordingly, the right wing of each fly was mounted in Euparal and retained; epidermal cell size was determined, in certain cases, by counting the number of bristles, each of which corresponds to a single cell, in a known area of the wing (Dobzhansky, 1929; Alpatov, 1930).

RESULTS

The culture techniques proved satisfactory and prepupae were obtained ranging in wet weight from 0·72 to 2·15 mg., compared with the range for the earlier work of 0·85–1·70 mg. Moreover, the ranges for the three types overlapped almost completely, being 0·79–1·80 for males, 0·72–2·01 for diploid females, and 1·18–2·15 for the triploid females. Of the 100 prepupae whose oxygen consumption was measured, the results for twenty-five had to be rejected: most of these were cases where the imago failed to emerge, but there were also a few where the flies unfortunately escaped before their type was determined. There was also one intersex. Thirteen males were included, and of the remaining sixty-two, twelve turned out to be triploid females and fifty diploid females. The surface area of forty-eight of the seventy-five was measured successfully, eight of them triploids.

A typical curve for the rate of oxygen consumption per mg. for a single prepupa is presented in Fig. 1: similar curves were obtained in all cases. It follows the expected course for this early stage of the prepupal period (Ellenby, 1938), the rate of oxygen consumption decreasing by between 30 and 40% during the course of the experiment. This agrees well with the previous finding. Of greater interest, however, is the level of oxygen consumption for the different classes of prepupae examined.

As similar curves were obtained in all cases, it is permissible to average, for each experiment, the rate of consumption for the whole experimental period. In Fig. 2 values for mean rate of oxygen consumption per mg. wet weight per hr. are plotted against body weight for all classes examined; Fig. 3 shows the values for oxygen consumption per sq.mm. of puparial surface. (Log. plots, for total oxygen consumption, total surface, and body weight, are shown below, in Fig. 4.) Values, on either standard, are somewhat lower than those already reported for the oxygen consumption of *vestigial* and *wild-type* prepupae (Ellenby, 1945*a*), the earlier mean value for oxygen consumption per sq.mm. of 0·426 comparing with 0·388 for the present series. In fact, the agreement between the series is reasonable, for the present value is based on experiments an hour longer and at a slightly lower temperature.

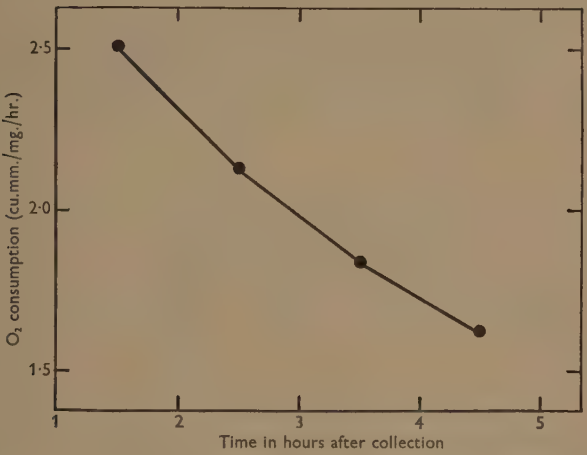

Fig. 1. Oxygen consumption of a single prepupa during the first hours of the pre-pupal period.

Oxygen consumption per unit of body weight falls off steadily with increasing weight; on the other hand, consumption per unit of surface is more constant. But, clearly, despite the variation in the results, and whatever the standard of comparison, there is little difference between triploids and the other classes. On either standard, more of the values for triploids, represented by closed circles, are below the line than above it, more so in the case of the weight standard. But there is little in it, all the triploid values being more or less 'in the thick' of the points for the other forms.

Transformation to a surface basis shows that the regression coefficient for oxygen consumption/sq.mm. and body weight, fitted by the method of least squares, is +0·0236 compared with +0·0141 obtained in the earlier work. Moreover, as the standard error of regression is ±0·01 the regression coefficient differs significantly from zero (P almost 0·02) showing that, unlike the earlier finding, oxygen

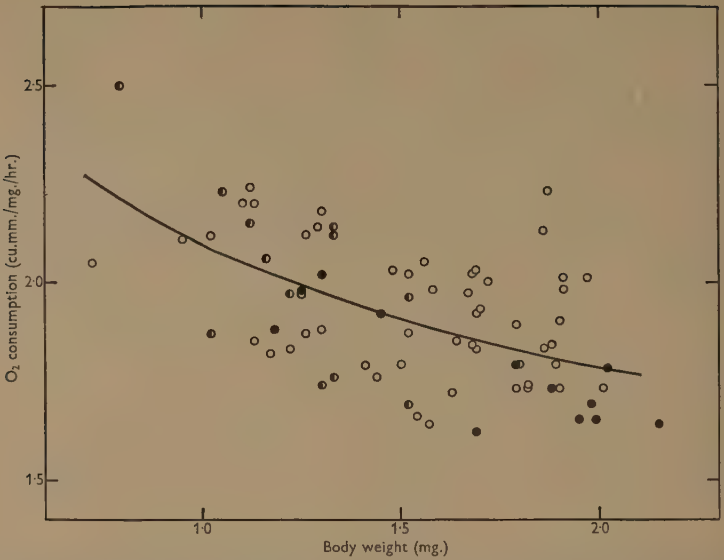

Fig. 2. Oxygen consumption and body weight for three types of prepupae. ○, diploid female; ◐, diploid male; ●, triploid female. Curve fitted by means of a logarithmic transformation.

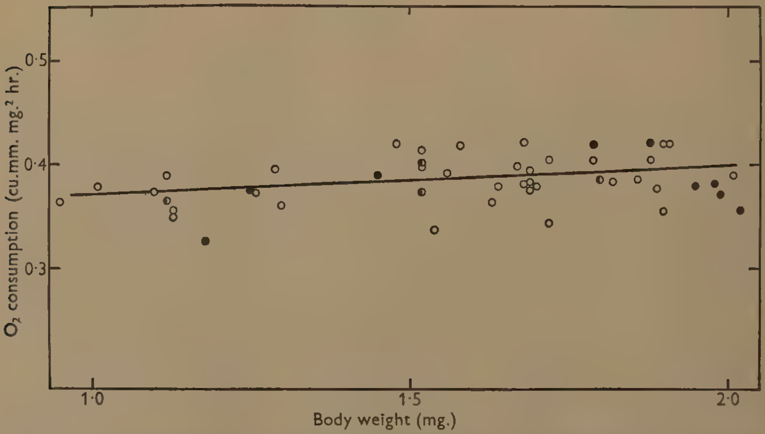

Fig. 3. Oxygen consumption and surface area for three types of prepupae. ○, diploid female; ◐, diploid male; ●, triploid female.

consumption per unit of surface varies significantly with the weight of the animal. The trend with increasing body size is small, amounting to a difference in oxygen consumption per sq.mm. between the extremes of the weight range of about 8%. Nevertheless, a straightforward comparison between mean values for the different classes of prepupae is out of the question. As a covariance analysis was necessary, it seemed desirable to carry it out with the more extensive data for oxygen consumption per unit weight.

The line fitted to the results presented in Fig. 2 was calculated from a logarithmic transformation of both variables. Covariance analysis with the logs. of the values showed that there were no significant differences between the regression coefficients for the different classes of prepupae; for the purposes of the diagram therefore, the curve was drawn from the pooled data. This showed that oxygen consumption per mg. was proportional to the −0·228 power of the body weight. As the weight ranges of the three types overlap almost completely, there is no objection to comparing the rates of oxygen consumption at the mean for all types.

For this the 'within lots' regression coefficient, a coefficient which is a weighted average of those for the separate classes calculated independently, provides the best estimate of the population regression. In fact, in the present case, its value, at −0·220, differs little from the value calculated from the pooled data; but it is the correct coefficient to use, and, as a good deal of experimental error is eliminated in this way, its standard error is very much smaller.

Table 1. *Comparison of adjusted means, logs.*

Type	Mean weight (mg.)	Mean O_2 (cu.mm./mg./hr.)	Adjusted mean O_2 (cu.mm./mg./hr.)	S.E. of estimate	Adjusted mean O_2 (cu.mm./mg./hr. antilog.)
Male	0·1113	0·2898	0·2739	±0·0126	1·879
Female	0·1908	0·2721	0·2737	±0·0058	1·878
Triploid female	0·2268	0·2491	0·2586	±0·0126	1·814

Mean values for oxygen consumption and body weight are calculated for each class, the difference of the mean class weight from the grand mean estimated, and then, by means of the 'within lots' regression coefficient, the adjusted mean value for oxygen consumption at the grand mean body weight is estimated; in effect, the class means are 'slid' along the regression line until they correspond to a common value for body weight. It is then possible to estimate whether there are significant differences between the adjusted mean values. The results of such an analysis are shown in Table 1. The test of significance is carried out with log. values as the regression analysis was carried out with a logarithmic transformation, but, for interest, the antilogs. of the adjusted mean values have been included. The agreement in adjusted mean values for male and female is astonishingly good, the two values only differing in the fourth place. Triploid females are slightly lower at 0·2586 (about 4% on the geometric means), but the standard error for difference from the others is ±0·0139 showing that the difference is very far from statistical significance. Clearly, the metabolic rates of diploids and triploids must be assumed to be identical.

In view of these findings an extensive investigation of cell size was hardly justified. However, Alpatov (1930) has shown that some slight increase in body size is probably due to increase in cell number: as Dobzhansky (1929) made no allowance for the body size of the animals used in his comparisons of triploid and diploid cell size, it was therefore necessary to ascertain whether, at any particular level of body size, triploid individuals had larger cells than diploids; I have satisfied myself that this is so. Particularly suitable material was provided by two specimens, one a diploid female (no. 49), the other a triploid (no. 76); this is presented in Table 2. The two animals have the same prepupal body weight, and the same puparial surface area. But although their wing dimensions are identical, the cells of the triploid are about one and a half times the size of those of the diploid; nevertheless, their rates of oxygen consumption are the same.

Table 2. *Comparison of diploid and triploid individuals of equal body weight*

Series no.	Type	Body weight (mg.)	Puparial surface (sq.mm.) area	O_2 consumption (cu.mm./mg./hr.)	Wing length (mm.*)	Wing width (mm.*)	Cells per 0·01 sq.mm.*
49	Diploid ♀	1·25	6·83	1·97	1·65	0·99	58
76	Triploid ♀	1·25	6·60	1·98	1·65	0·99	40

* Dimensions as defined by Alpatov, 1930.

DISCUSSION

The results clearly show that, after adjustment for differences in body size, there are no differences in the rates of oxygen consumption of triploid female prepupae and either diploid male or female. As triploids have larger cells than diploids of the same body size, cell size has no influence on the general level of metabolism.

The values for oxygen consumption and body weight were used in the analyses which led to the above conclusions rather than values relating oxygen consumption and surface area because they were more extensive; owing to casualties in the measurement of surface area there were only forty-eight values as compared with the seventy-five for oxygen consumption and body weight. However, Fig. 4 shows that the forty-eight surface and oxygen values would have given almost as much information as the seventy-five!

Fig. 4*a*, *b* are double logarithmic plots of the values for total oxygen consumption and total surface area (*a*), and total oxygen consumption and body weight (*b*), for the forty-eight animals for which all three values are known: the scales for oxygen consumption are identical, and the scales for the other two variables have been made as equal, relatively, as possible. The agreement with the fitted line is clearly better in the case of the surface relationship but perhaps not so markedly superior as is shown by the regression analyses. After subtracting the variance attributable to regression, the error mean squares derived are 0·000647 for the oxygen surface relationship, and 0·00084 for the oxygen weight relationship. The error variance of the former is therefore three-quarters of the latter and the forty-eight surface oxygen values give as much information as sixty-four oxygen weight values!

I have already had occasion to point out that a better prediction of the oxygen consumption of the littoral isopod *Ligia oceanica* could be obtained from the square of a fairly inaccurately determined linear dimension than from an accurate determination of body weight (Ellenby, 1951). However, in the present case the animals were reared under very constant culture conditions and spent their entire lives at one temperature; moreover, they were weighed after they had spent 5 hr. in the constant atmosphere of the respirometers. It is therefore most remarkable that, even under such constant conditions, a relatively inaccurate surface-area measurement should enable a better prediction of oxygen consumption to be made than an extremely accurate determination of body weight. Hitherto it has been thought that oxygen consumption is proportional to some function of body weight and that the relationship of the former to surface area was due to the relation of surface area itself to body weight; these findings suggest that, in the present case at least, the reverse may be the case; however, a more detailed investigation of this and the *Ligia* material is in progress.

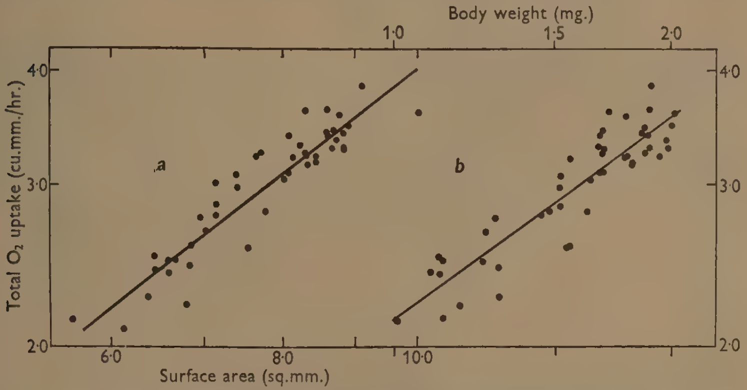

Fig. 4. *a*, total oxygen consumption and total surface area; *b*, total oxygen consumption and body weight. All on double logarithmic grid with comparable scales.

The results of the present investigation differ very markedly from those presented in 1945, for in the present case, the rate of oxygen consumption per unit weight decreases far less rapidly with increasing body size. The two series of values are plotted in the same diagram in Fig. 5, closed circles being used for the earlier series. The series are not exactly comparable; each experiment of the present series lasted longer, reducing the mean value somewhat, and the temperature at which the measurements were carried out was not quite the same. Nevertheless, these factors would presumably affect the general level of the curve relating rate of oxygen consumption and body weight rather than its slope; in fact, it is clear from the figure that the slopes are markedly different.

The curves presented in Fig. 5 were calculated from the data of each set of results using a logarithmic transformation; they are the lines which fit the two sets of data best if each set is considered to be one population. Each curve depicts the trend of the points very well. The regression coefficients in the two cases for the relationship of oxygen consumption per mg. and body weight are −0·446 and −0·228 for the earlier and present series respectively; total oxygen consumption would then be proportional to the 0·554 and 0·772 power in the two cases. An analysis of variance showed that the difference between the regression coefficients is highly significant the ratio between the appropriate mean squares, being 8·42 giving a value for *P*, the probability, of 0·01. However, treating each set of values as one population is not strictly correct; the earlier series includes results for five types of animals, namely *vestigial* male and female, *wild-type* male and female, and the male heterozygote, and the present series is based on *yellow*$_2$, *vermilion*, *forked* male, female, and triploid female. Even though the analyses showed that there were no differences between the regression coefficients for each category calculated separately, the best estimate for the population trend for each series is given by the

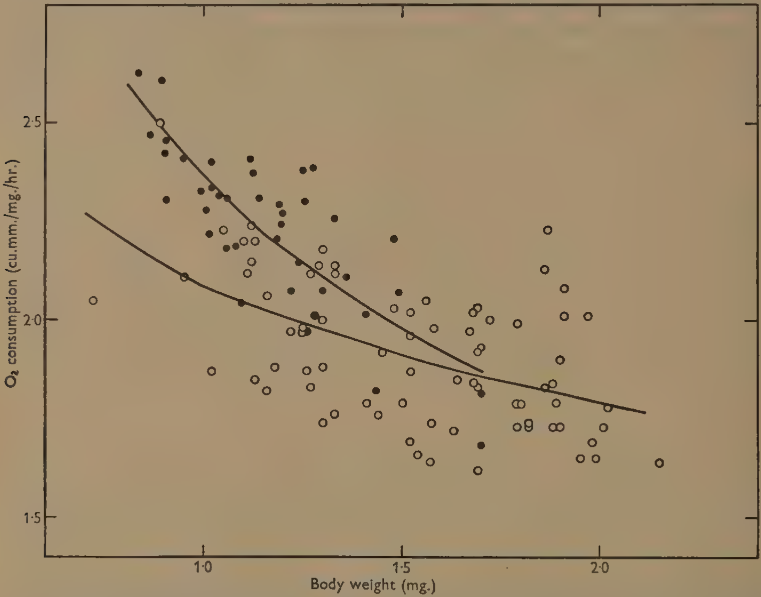

Fig. 5. Oxygen consumption and body weight for two series of experiments. ○, present series, all types; ●, earlier series (Ellenby, 1945*a*), all types. Curves fitted by means of logarithmic transformations.

'within lots' regression coefficients. These were accordingly evaluated; they showed little difference from the values already presented, being −0·475 for the earlier series and −0·220 for the present series compared with −0·446 and −0·228 respectively, slightly increasing the disparity. As the stricter analysis eliminates a good deal of experimental error, the standard error of regression in each case is very greatly reduced, actually to a small fraction of its former size. Whereas the difference between the original regression coefficients was highly significant, with the more precise test it is overwhelmingly so. And all coefficients, precise or less precise, differ significantly from the −0·333 expected on the basis of a 2/3 power relationship; the value for the earlier series shows that oxygen consumption per unit weight falls off more rapidly, while that for the present series shows it to fall less rapidly than such a relationship would lead one to expect. These, and other relationships are summarized in Table 3.

Table 3

	Present series	Ellenby, 1945*a*, *b*
(1) Regression coeff. of O_2/mg. on body weight	−0·220	−0·475
(2) S.E. of regression of (1)	±0·0022	±0·0044
(3) Relation of total O_2 uptake to body weight	+0·780	+0·525
(4) Regression coeff. of O_2 uptake/sq.mm. on body weight	+0·0236	+0·0141
(5) S.E. of regression of (4)	±0·010	±0·016
(6) Regression coeff. of surface/mg. on body weight	−0·431	−0·485
(7) S.E. of regression of (6)	±0·054	±0·034
(8) Relation of total surface to body weight	+0·569	+0·515

My earlier paper (Ellenby, 1945*a*) showed that oxygen consumption was proportional to surface area; surface per mg. bore the most unusual relationship to body weight of the −0·485 power, that is, total surface was proportional to the 0·515 power of body weight (Ellenby, 1945*b*); the unusual nature of these values is discussed in the original paper and more recently (Ellenby, 1951); although '2/3' is seldom obtained, it is rare in the case of either the surface area or oxygen consumption relationship for values to differ from it so much. Nevertheless, the agreement between the regression coefficients for puparial surface area and prepupal oxygen consumption, each in relation to body weight, was very close indeed and the conclusion that oxygen consumption was proportional to surface area gained strength, if anything, from the agreement between two such unusual values. However, the more conventional values obtained in the present case for the oxygen relationship (see Weymouth *et al.* 1945, Kleiber, 1947, Ellenby, 1951), 0·78 for total, and −0·220 for consumption per unit weight, reduces the comfort derived from the mutual support of two atypical values: further inquiry was necessary.

Values for total puparial surface area for the animals examined in the present investigation are presented in Fig. 6: there is reasonable agreement among the data.

Regression analysis showed that surface area per mg. was proportional to the $-0{\cdot}431$ power of body weight, or total surface, as presented in the diagram, to the $0{\cdot}569$ power. Agreement with the earlier values of $-0{\cdot}482$ and $0{\cdot}518$ is reasonable; as might be expected, the two sets of values do not differ significantly; in fact, an analysis of variance showed that the mean square for error was more than twice as great as the mean square appropriate to the difference between the coefficients. The present value, unlike the earlier one, does not differ significantly from a '2/3' relationship, but, of greater interest is the fact that it does differ significantly from the $-0{\cdot}228$ power which was found to relate oxygen consumption per unit weight to body weight; with a standard error of regression of $\pm 0{\cdot}054$, P is almost $0{\cdot}001$. The finding that oxygen consumption per unit surface is not constant over the size range (Fig. 3) is thereby confirmed.

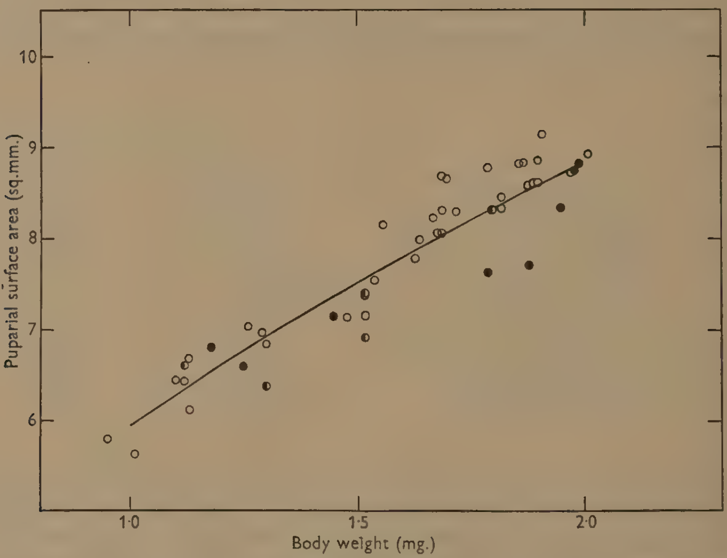

Fig. 6. Puparial surface area and body weight. ○, diploid female; ◐, diploid male; ●, triploid female. Curve fitted by means of logarithmic transformation.

The relationship between surface area and body weight is reasonably similar for both sets of determinations; both coefficients relating surface and weight are atypically low and there is no significant difference between them. It is possible, however, that there is some fault in the technique tending to give low surface area measurement for large animals. Measurement of 'surface area' is at best an approximation justifiable only so long as the approximation is similar over the size range. If there were a progressive error of this sort in the method, 'surface' would appear to increase less rapidly than it should, thus giving a low coefficient.

The puparial surface area is measured after the empty puparium is compressed between slides. During the process, a certain amount of distortion takes place, and, clearly, if the relative distortion increases with the size of the animal, a progressive error would be introduced. Now although there is distortion in the compressed puparium, the anterior end is completely undistorted. Part of the anterior end of the puparium is modified into the operculum through which the imago emerges (Strasburger, 1935); it therefore always splits in exactly the same way, the portions previously united to the lateral margins of the operculum being laid out to each side. A comparison of the area of this undistorted portion with that of the whole puparium would therefore be of interest.

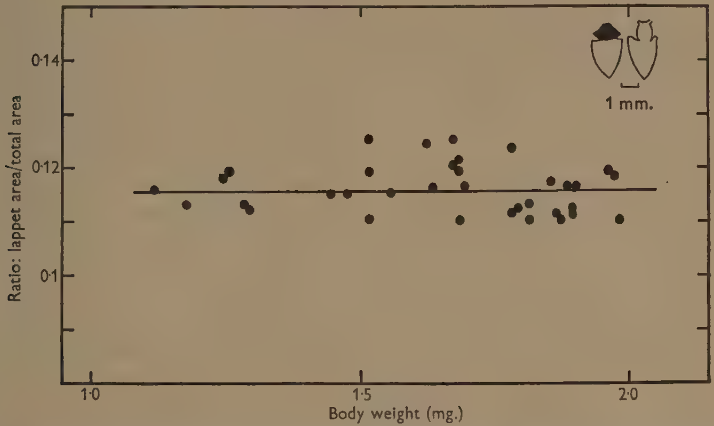

Fig. 7. Inset shows the outline of the projected flattened puparium; black portion is the undistorted anterior, 'lappet', portion. Diagram shows the relationship of the area of this undistorted portion to the area of the whole. The line fitting the data does not differ significantly from the horizontal showing that the degree of distortion produced by the flattening process is approximately constant over the size range.

In Fig. 7 the ratio of the two areas has been plotted against body weight for the thirty-eight cases where the comparison was possible; the areas concerned are the whole area of the inset of Fig. 7 and the black portion alone. Clearly there is no tendency for the values for this relationship to vary with the size of the animal; in fact, the regression coefficient is only +0·000028 compared with a mean value of 0·116. The demonstration is not complete proof that there is no progressive change in the degree of distortion, for the ratio would also be constant if there were an increase in the relative area of the undistorted portion at the same rate as an increase in the distortion of the remainder; but such a coincidence seems unlikely. It seems reasonably certain, therefore, that the comparatively low value for the exponent relating surface area and body weight is a true representation of the relationship and is not due to technical faults.

In both sets of data, then, surface area varies as some power of the body weight round about 0·55. In the earlier series, oxygen consumption varied at the same rate with increasing body size, while in the present series it was found to vary as the 0·78 power. The discrepancy is most surprising—and most intriguing. Values for the exponent of body weight vary for different animals, but the differences are comparatively slight. In the present case, not only is the difference between the two values large, but it is observed in animals belonging to the same species and, of exactly the same stage. As in the case of the surface area measurements, the simplest explanation would be that the discrepancies were due to faulty experimental procedure; but it is difficult to envisage a technical fault which affected the rate of oxygen consumption progressively with increase in body size. If the low value for the weight exponent had been obtained in the present, rather than the earlier series, one might have thought that a condition of oxygen lack was perhaps built up by the more heavily respiring larger animals in the comparatively restricted volume of the micro-respirometers; there is no basis for such a possibility in the earlier experiments. Moreover, a comparison of the two curves of Fig. 4 would, if anything, add confidence to one's support for the earlier, and atypical, value; the scatter is less and the values follow the trend with considerable fidelity. This is borne out by a comparison of the standard errors of estimate for the regression analyses of log. oxygen consumption per mg. on log. body weight; for the earlier series the value is ±0·0014 on a mean value of 0·345 compared with the corresponding values in the present series of ±0·0015 and 0·279. The earlier series at least holds its own.

The earlier experiments were carried out on groups of prepupae and not with single animals as in the present series. As I have already pointed out (Ellenby, 1945*b*), since oxygen consumption is not proportional to weight itself, the total weight of a group of animals will not give a fair estimate of its metabolic potentialities; two groups of the same number of individuals may have the same total weight, but if the size distribution in the two groups is not identical, the total oxygen consumption of the two groups will differ. Moreover, if the distribution is skew in opposite directions for groups of small and large animals, the shape of the curve relating oxygen consumption to body weight will be affected. If groups of small mean weight have an abnormal number of small animals and there is also a tendency for groups of large mean weight to have a non-normal number of large animals, the mean rate of oxygen consumption will be higher than expected for the group of small mean weight, and lower for the group of large mean weight: oxygen consumption per unit weight would then appear to decrease more rapidly than it should. The possibility seemed unlikely, but worthy of consideration in the two series. Examination of the sizes of the puparia making up the groups used in the original surface-area measurements, however, showed them to be reasonably homogeneous and certainly without the progressive change required. Admittedly, these groups are not those used in the original oxygen consumption measurements but they were collected under exactly the same conditions and are undoubtedly comparable, certainly from the present point of view.

But working with groups will have another and, theoretically, more serious aspect, for it can be shown mathematically* that, if rate of oxygen consumption follows a 'power law', 'mean' oxygen consumption per mg. based on the total consumption of a group of animals will always be less than the true mean based on the values for the individual animals; furthermore, this effect will vary with the absolute size of the animals concerned and so alter the *shape* of the curve relating oxygen consumption and body weight. Fortunately these effects will be small if the groups are reasonably homogeneous, which was almost certainly the case; but it seemed desirable to estimate the possible magnitude of the effect.

In order to test the effect of grouping, the results for the present series of experiments with individual prepupae were arranged in arbitrary groups. For any particular day, the animals would correspond, more or less, with a group used in the earlier series. Groups were kept homogeneous as to type, and animals differing greatly in size were not included in the same group, as such animals undoubtedly did not occur in the groups of the earlier series. Nevertheless, there was a fair variation: for example, the first group contained animals of mean weights 1·97, 1·91, 1·26, 1·69, 1·90, 1·88, and 1·82 mg. The regression coefficient calculated from these synthetic groups with a log. transformation of the values for both mean oxygen consumption and mean weight showed that oxygen consumption per unit weight was proportional to the $-0·261$ power of the body weight or total consumption to the 0·739 power. These values are little different from those arrived at with the individual items.

There are a number of cases in which the exponent relating oxygen consumption and body weight has been shown to vary for different parts of the weight range (Michal, 1931; Zeuthen, 1947) and for animals raised on different diets (Teissier, 1931). These help little in the present instance for the animals of both series of experiments were raised under identical conditions, and, unlike the other cases, were compared at exactly the same stage of growth. Moreover, Zeuthen's claim that oxygen consumption varies directly with body weight up to 1 mg., only decreasing for animals beyond this size, is also of little help; although the animals of the first series of experiments were, on the whole, of slightly lower weight range than the later series, they are almost all above 1 mg., and, moreover, their oxygen consumption decreases more rapidly than that of the later series. The conclusion that the differences in the exponents obtained in the first and second series are due to differences, not necessarily genetical, in the animals used in the experiments seems inescapable. As, despite their genetical differences, the two sets would have been considered far more uniform than those generally used in metabolic studies, an understanding of the reasons for the different values would probably help to explain why, in fact, metabolic rate does vary with the size of the animal. The results certainly suggest that it is most unwise to assume any particular value for the exponent for a particular uninvestigated case.

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* I am very grateful to Dr D. A. Evans for demonstrating this to me.

SUMMARY

1. The oxygen consumption and surface area of individual diploid and triploid prepupae of *Drosophila melanogaster* have been measured, the cells of triploid animals being larger.

2. The mean weights for the types examined are different but their ranges overlap almost completely. By covariance analysis it is shown that, after adjustment for difference in body size, there are no differences in the rates of oxygen consumption. It is concluded that, for these animals, cell size has no influence on the rate of oxygen consumption.

3. The relationships between body weight, surface area, and oxygen consumption have been further investigated. It is shown that, despite the greater inaccuracy of the method by which surface area is determined, oxygen consumption can be predicted more accurately from surface area than from body weight.

4. The results are discussed in relation to an earlier investigation of the oxygen consumption of other genotypes (Ellenby, 1945*a*, *b*). Possible technical causes of certain differences between the two series of results in the relationship of oxygen consumption and body weight are explored; it is concluded, however, that they are almost certainly due to differences, not necessarily genetical, between the animals used in the two series.

The experiments on which this paper is based were carried out in 1938 in the Department of Zoology, University College, London. I am most grateful to Dr Sarah B. Pipkin for the patience with which she instructed me in the techniques of handling a triploid stock.

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METABOLISM OF THE BLACK SNAKE EMBRYO

I. NITROGEN EXCRETION

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(*Received* 6 *December* 1952)

Very few data exist on the nitrogenous excreta of reptilian embryos. Tomita (1929) indicates a substantial urea excretion by the turtle *Chelonia*, but no data are available for saurian reptiles. They have been presumed (Fiske & Boyden, 1926) to excrete uric acid because 'a substance as soluble and diffusible as urea could not possibly replace it (uric acid) as an end product when the organism and its excretions are confined to a closed system, the walls of which are only permeable to matter in the gaseous state'. Needham (1931) estimates that the chick urea concentration would reach 165 mg. % if it excreted only urea, and intimates that such an accumulation in a cleidoic egg would be pathological.

The author has therefore sought data which will supplement our knowledge of metabolism of those vertebrate embryos whose progenitors were most directly concerned with the process of evolving the cleidoic egg among the vertebrates; such data may, indeed, suggest details of the mechanics of some excretory enzymes operative in adults. Finally, the data reported below will provide comparison with excretory details of viviparous saurians, whereby the role of the placenta in embryonic excretion may be evaluated.

MATERIAL AND METHODS

Data were accumulated on ammonia, urea and uric acid production during the incubation period of two clutches, totalling thirty-three eggs, of the black snake, *Coluber c. constrictor*. The egg is leathery firm, creamy white, and usually approaching the form of a prolate spheroid; mean volume of all eggs was 8·6 ml., mean weight 9·3 g. and mean axial measurements were 36·6 × 21·4 mm. The developmental period in the laboratory was 68 days, 25 June–1 September.

Ammonia was measured by the Conway microdiffusion technique (1947), as gas either effused through the shell or liberated from solution by K_2CO_3. Urea was measured similarly as the amount of ammonia liberated by urease in Conway microdiffusion dishes and determined titrimetrically. Uric acid was measured colorimetrically according to the method of Brown (1945). Egg contents were subdivided in the following way for each determination: albumen, yolk plus yolk-sac, embryo, amniotic fluid and chorio-allantoic membrane. Weights reported below were made on a Christian Becker analytical balance to the nearest mg. Eggs

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* The author is grateful to Prof. C. A. Kind for many valuable suggestions, and to Mr Robert E. Beyer for making the uric acid assays reported herein.

were incubated at room temperature, whose extremes were 21–30° C.; the incubation chamber was a large covered culture dish, in which the eggs were laid on cheesecloth or filter-paper. It is necessary that this bed be kept moist, since a water-saturated atmosphere alone does not appear to supply sufficient water to the egg. This material was occasionally extracted with distilled water and assayed for urea. Early embryos were homogenized with a Potter homogenizer, and later ones were minced and ground with mortar and pestle and diluted with a quantity of water suitable to embryonic size. Yolk and yolk-sac were diluted with 50 ml. H_2O and were stirred vigorously for several minutes and allowed to stand for 10 min., after which an aliquot was taken for urea determination. Samples of albumen were taken in 0·2 ml. pipette, and during later stages, when concentration of excreta was greater, these were diluted to 2·0 ml. for urea and uric acid determinations; the remainder of the albumen in the egg was measured volumetrically. The assays for liver and kidney urease were made by the Conway technique, using as substrate 0·5 ml. of 25 mg. % urea.

RESULTS

Certain progressive gravimetric and volumetric changes of egg contents are presented in Table 1. These will be related to excretory data presented below.

Table 1. *Increase in mass of the embryo and membranes and decrease in yolk content*

Age (days)	Weight (g.)			Volume albumen (ml.)
	Embryo	C-A	Yolk-sac + yolk	
1	0·005	—	—	—
11	0·50	—	6·76	0·3
12	0·47	—	6·85	0·3
18	0·65	—	7·10	0·5
20	0·92	—	7·60	0·6
24	1·00	0·14	6·06	1·2
27	1·47	—	6·63	1·4
31	1·86	0·32	5·66	1·0
34	1·70	0·15	5·26	1·6
43	2·49	0·27	4·95	1·3
45	3·46	0·26	4·05	0·9
51	3·86	0·21	3·88	1·1
52	4·39	0·20	3·54	2·5
58	5·86	0·30	2·46	1·9
62	5·78	0·20	1·96	2·0
67	6·85	0·20	0·92	1·9

C-A = chorio-allantoic membrane.

Growth. Growth of the embryo, yolk-sac and chorio-allantoic membrane is shown in Fig. 1, from which it is apparent that embryonic and yolk-sac weights are approximately equal until the 18th day. Although at a later stage the rate of increase of embryonic weight greatly exceeds that of the yolk-sac, the latter does increase until the 58th day. The first record of weight of the chorio-allantoic membrane was made on the 24th day; actual specification, therefore, of time of origin of the allantois cannot be made, though conjecture on the basis of those data which are shown in Fig. 1 would place its origin at about the 10th day. Characteristic features of the growth of the embryo are shown also in Fig. 2 in which it is apparent

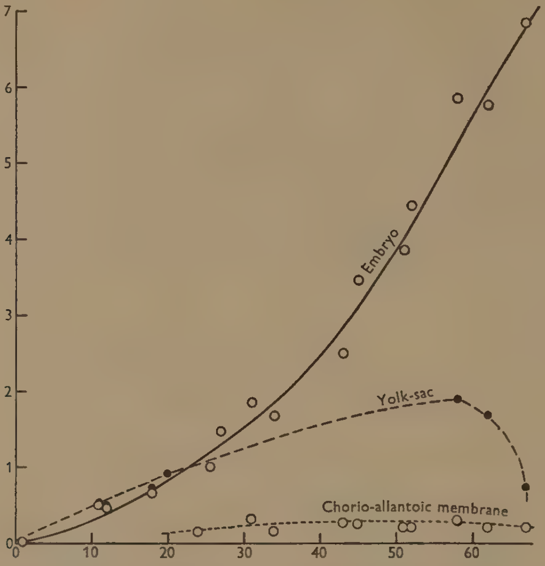

Fig. 1. Growth of embryo, yolk-sac and chorio-allantoic membrane. Ordinate: g. (wet weight). Abscissa: age, days after deposition.

Table 2. *Synopsis of* k *values for variables in black snake development, calculated from terminal points of theoretical exponential curves for periods listed*

Embryonic weight	0·46, 1–11; 0·057, 11–34; 0·039, 34–67
Total nitrogen	0·31, 1–11; 0·062, 11–67
Ammonia	0·27, 1–9
Urea, total	0·047, 11–34; 0·040, 34–58; −0·027, 58–67
Urea, yolk-sac	0·057, 11–20; 0·0, 20–58
Urea, conc. embryo	0·029, 20–58; −0·105, 58–62; 0·0, 62–67
Urea, conc. yolk-sac	0·061, 11–18; 0·024, 18–67
Uric acid	0·171, 58–67
Yolk and yolk-sac (weight)	−0·013, 11–51; −0·072, 51–67

that there are three distinguishable growth phases. (With reference to the growth equation, $W = Ae^{kt}$, k values are recorded in Table 2.) Further comment on embryonic growth will be made in connexion with the results on excretion. Of particular interest with respect to egg contents is the progressive increase in volume of 'albumen', an apparently paradoxical phenomenon, resulting from continuous uptake of water as the amount of stored urea increases. Although amniotic fluid was present during the first half of the developmental period in the amount of 0·5–1·0 ml., it is omitted from the tabulation because of the virtual impossibility of measuring amniotic fluid in later stages when it constitutes hardly more than a film on the surface of the embryo.

The most careful dissection did not permit identification of allantoic fluid at any time during development, an observation in sharp contrast with the situation in avian development. The absence of allantoic fluid is apparently correlated with the increasing volume of albumen and the function of the latter as a nitrogenous waste reservoir.

Excretion of nitrogenous waste. A summary of excretory products is presented in Table 3 and each component will be reported upon separately.

Table 3. *Cumulative nitrogen excretion by the black snake embryo, in mg nitrogen*

Age (days)	Ammonia (gas)	NH_4^+ salts	Urea	Uric acid	Total
1	0·24	—	—	—	0·24
2	0·48	—	0·014	—	0·49
3	0·87	—	(0·11)	—	0·98
4	1·17	—	—	—	—
5	1·43	—	(0·26)	—	1·69
6	1·77	—	—	—	—
7	2·28	—	(0·45)	—	2·73
8	2·62	—	—	—	—
9	2·67	—	(0·65)	—	3·32
11	—	—	1·24	—	—
12	(2·67)	0·075	1·33	—	4·08
18	(2·67)	0·160	2·14	—	4·97
20	(2·67)	0·400	2·43	—	5·50
24	(2·67)	0·10	2·97	—	5·74
27	(2·67)	0·10	3·19	—	5·96
31	(2·67)	0·10	3·34	0·16	6·27
34	(2·67)	—	4·36	0·18	7·21
43	(2·67)	—	5·09	0·15	7·91
45	(2·67)	—	4·93	0·17	7·77
51	(2·67)	—	5·81	0·17	8·65
52	(2·67)	—	7·36	0·07	10·10
58	(2·67)	—	9·36	0·56	12·59
62	(2·67)	—	8·16	0·90	11·73
67	(2·67)	—	7·32	2·56	12·55

Ammonia. Ammonia is the predominant excretory product during the first 10 days of development, and during this period it is released from the egg as a gas. Coincident with the transformation of excretory devices to urea production, there is a decline in ammonia formation. Some ammonia continues to be formed until the 20th day. This appears exclusively as NH_4^+ salts and is stored only in the yolk. Decline in ammonia production coincides with the development of the chorio-allantoic membrane, although a functional relationship is not hereby implied. The salts excreted to the 20th day must therefore have been excreted by the yolk-sac. Moreover, the disappearance of NH_4^+ salts from the yolk after this time implies a reabsorption and presumably conversion to urea.

Urea. Urea is produced in minute quantities at the beginning of development, and at approximately the 12th day is synthesized in greater quantity than ammonium salts. The production of urea is shown graphically in Fig. 2 in relation to total nitrogen excreted and to uric acid (the semilogarithmic plot of embryonic growth is reproduced in this figure for ease in relating nitrogen excretion to growth). Two aspects of urea elimination are apparent: (1) that it accounts for a very large percentage of the total excreted N, and (2) that the amount excreted declines after the 58th day. In terms of excreted nitrogen the decrease in urea after the 58th day is 2·04 mg., the increase in uric acid 2·00 mg.—values sufficiently close to suggest quantitative relationship.

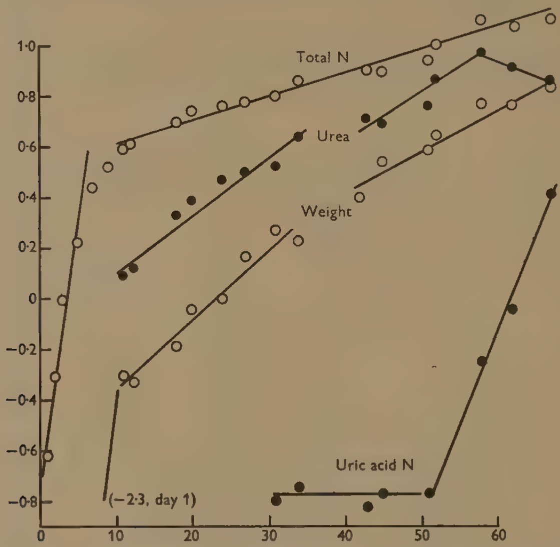

Fig. 2. Semilog. plot of embryonic weight increase (g.) and excreted total nitrogen, urea, uric acid nitrogen (all in mg.). Abscissa: age, days after deposition. For *k* values, cf. Table 2.

The time periods for calculation of *k* do not coincide with those related to embryonic growth. Hence for urea production, $k = 0{\cdot}047$ (11–34 days), 0·040 (43–58 days) and corresponding to the terminal decline in urea content, −0·027 (58–67 days).

Relationship of urea production to changes in the yolk-sac is shown in Fig. 3, in which the rate of utilization of yolk is also depicted. The urea content of the yolk and yolk-sac reaches a peak at approximately 20 days; but it is also obvious in this figure that the concentration in the yolk continues to increase even beyond the time when the total urea content of the egg declines. This increase is accounted for between days 52 and 62 by decrease in amount of yolk; the slight decrease in urea content during this interval would then be assigned to reabsorption from the yolk to the vitelline circulation. After day 62 the concentration on the basis of decrease in weight alone (now almost entirely decrease in weight of the yolk-sac) would be 600 mg. %. That it is actually 153 mg. % implies clearly a rapid and selective release of urea from the yolk-sac itself during the terminal 5-day period. This is facilitated by, and perhaps contingent upon, a decline in concentration in the embryonic tissues from 120 mg. % on day 58 to 62 mg. % on day 67.

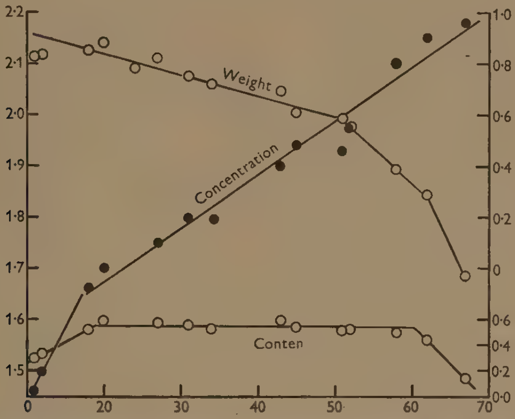

Fig. 3. Semilog. plot showing decline in weight of yolk plus yolk-sac (g.), urea content (mg.) throughout development and concentration of urea in yolk and yolk-sac (mg. %). Ordinates: left scale, log., mg. %; upper right scale, log., g.; lower right scale, log., mg. urea. Abscissa: age in days after deposition.

The decline in weight of yolk and yolk-sac is described by a k value of $-0{\cdot}013$ to day 51, whereas the urea concentration increases at twice this rate ($k = 0{\cdot}024$). It would therefore appear that the urea-synthesizing activity of the yolk-sac diminishes after day 52 and ceases by day 62. Cessation of urea synthesis is temporally correlated with the rise in uric acid production.

The observation of decreasing content and concentration in the embryonic homogenate, contemporaneous with the decline in content in the yolk, led to a test for urease within the embryo. Samples of liver and kidney, when incubated with 25 mg. % urea, released ammonia in significant quantities. Control samples, incubated in phosphate buffer without substrate, but subsequently treated with 1·0 ml. K_2CO_3 (routine procedure to test for NH_4^+ salts) were negative.

In actual fact, 8·0 mg. kidney and 16·0 mg. liver each released 7% of the total ammonia present; in a second trial, using larger tissue samples, 50 and 70% respectively of the ammonia present were released by kidney and liver. These data, although not of quantitative significance, would seem to supply evidence for the mechanism which could be responsible for the conversion of reabsorbed urea to another compound.

Although concentration of urea in the yolk exceeds 150 mg. % (almost exclusively in the yolk-sac in the last measurement) and in the embryo reaches 120 mg. %, neither of these locations is the principal storage place of urea. It will be observed in Fig. 4 that concentration in the albumen reaches 500 mg. %, and the quantity stored here is approximately 55% of the total. Upon hatching, the embryo leaves the albumen in the egg, whereas in birds its absorption has been observed by Romanoff (1953) and Witschi (1949) who describe the path of absorption. The requirement of water during incubation had previously been observed by the author for snakes (1952), but no specific functional significance could be attached; the continuous rapid increase in dissolved urea suggests that it is primarily used as a solvent for the latter and accounts for the progressive increase in 'albumen' volume.

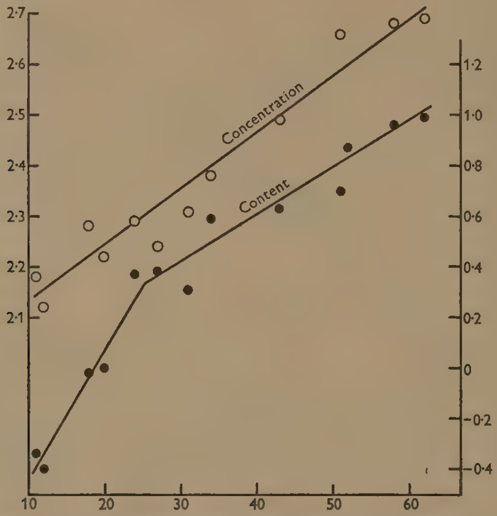

Fig. 4. Relationship of urea content to urea concentration in albumen. Ordinates: left scale, log., mg. %; right scale, log. urea content in mg. Abscissa: age, days after deposition. Contrast with curves shown in Fig. 3.

Aqueous extraction of filter-paper or cheese-cloth serving as a bed for the eggs and subsequent assay for urea was entirely negative. It is clear, then, that the shell membrane is impervious to urea and that the urea measured constitutes the total amount produced by the embryo.

Uric acid. It is apparent from Fig. 2 that uric acid is a negligible factor in excretion of nitrogenous waste in the black snake embryo during the first 3/4 of the developmental period. Excreted uric acid constitutes only 20% of the total nitrogenous waste, but accounts for nearly 100% of excreted nitrogen during the last 10 days within the egg. Separation of parts of the egg was made for uric acid determination, but the quantity present in parts other than the albumen was negligible even during the last week. Concentration, however, was high in the chorio-allantoic membrane.

Because of the low titre of uric acid in embryonic homogenates throughout development, and because of the high concentration in the chorio-allantoic membrane, it is presumed that the latter is the principal site of synthesis. This would imply that the ammonia released by urease in the foetal liver or kidney is not (at least completely) integrated into the uric acid molecule intra-embryonically. Once produced, it is excreted into the albumen by the membrane. This finding requires that uric acid after synthesis pass through the mesodermal, vascular layer of the allantois. Such a mechanism is at variance with allantoic function in the bird, in which uric acid remains on the entodermal side of the allantois.

Total nitrogen. The form of the curve of total nitrogen production (Fig. 2) from the first post-deposition day to the 10th, and the similarity between its k value (0·31) and that of ammonia (0·27) underline the fact of primary ammonia secretion during this period. Since both k values are substantially lower than that of growth during a comparable period (0·46) it would appear that protein is not the sole energy source at this time. However, during the remainder of the developmental period the rate of nitrogen excretion (0·062) is higher than that of growth (0·057, 0·039), implying a negative nitrogen balance and presumably indicative of the use of protein not only for protoplasmic synthesis, but for energy production as well. Fukuda (1939) finds a protein-fat ratio of approximately 4 : 3 in newly laid eggs of twelve species of oriental snakes; this ratio is somewhat higher than that of the chick as reported by Romanoff & Romanoff (1949). Protein and fat analyses of the yolk and embryo homogenates now in progress may aid in resolving this question.

The high initial rate of nitrogen excretion reported may possibly be exaggerated for the following reason: mating time of these snakes is commonly mid-April, and fertilization is estimated to occur early in May; hence an intra-uterine developmental period of 6–7 weeks intervenes, during which time a mechanism for voiding excreta is not available (since urea is not yet produced in quantity). The ammonia produced during this period may be stored, perhaps as ammonium salts, perhaps as glutamine. Consequently, gaseous ammonia released after deposition of the eggs should be assigned in part to the previous 6 weeks of development, rather than to the embryonic mass increase shown in Figs. 1 and 2. Thus the total nitrogen measured is quite accurate for the entire developmental period, but it is misleading to relate it only to the post-deposition growth.

Nitrogen excretion in viviparous forms. Placental snakes might be expected, *a priori*, to excrete urea since storage need not constitute a problem. Although this aspect of reptilian excretion has not been explored fully, a few data are at hand which corroborate this hypothesis. Analysis of embryos of the garter snake, *Thamnophis s. sirtalis*, approximately a week prior to parturition is reported in Table 4.

Perhaps more revealing is the fact that the total amount of urea recoverable in the embryo and adnexa of the garter snake at this time is 1·44 mg., whereas in the black snake the total approaches 20·0 mg. The discrepancy in embryonic mass (the garter snake embryo is about one-fourth the size of the black snake) is not sufficient to account for the urea differences between the two. The suggestion is plainly that the garter snake embryo does excrete urea through the placenta. Although this conclusion is supported by a limited number of analyses of maternal blood prior to parturition (mean = 10·1 mg. %) and after parturition (mean = 5·3 mg. %), more data are required to establish the method and quantity of excreted urea during pregnancy.

Table 4. *Concentration of urea in mg. %*

	Embryo	Yolk	Placenta	Albumen
Black snake	62·0	153·0	220·0*	500·0
Garter snake	63·0	25·9	44·4	95·0

* Chorio-allantoic membrane.

DISCUSSION

The mechanism here proposed for nitrogen excretion is the following: initial excretion of ammonia as a gas, continued excretion as ammonium salts in progressively diminishing quantity, correlated with increased excretion of urea. In this latter respect the yolk-sac is initially of cardinal importance and continues its function until disappearance of the yolk. As the allantois develops, it becomes progressively more involved in urea excretion, with the albumen serving as its reservoir. The urea stored in the yolk and yolk-sac is reabsorbed by the vitelline circulation, and during the last 10 days of development would then be converted within the embryo to ammonia; the latter is then incorporated in uric acid probably by the allantois, and voided into the albumen.

The succession of excretory products here described is like that of the bird; the relative importance of urea is, however, in sharp contrast with the predominantly uricotelic metabolism of the bird. The time during the incubation period of attainment of uricotelism in the black snake embryo and its relationship to urea both tend to shed light on the origin of this aspect of the metabolism of cleidoic eggs. Uric acid formation in significant quantities clearly is contingent upon concentration of urea in the tissues; and this concentration may serve as a trigger mechanism to stimulate uric acid synthesis.

Of additional interest is the considerably negative nitrogen balance as evidenced by the fact that *k* values for total nitrogen excretion are higher than for growth (wet weight) after the first 10 days. This points not only to the use of protein as an energy source during the latter part of development but possibly as well to a rather inefficient conversion of stored protein to protoplasm. The thesis of Needham that carbohydrate is the initial source cannot be denied since pertinent data must come from the period of development prior to deposition of the egg. Fat may well be the principal source of energy during the latter part of development, but almost certainly protein contributes to an extent greater than the 5% characteristic of the chick.

With respect to (1) requirement of water, (2) use of protein as an energy source, (3) excretion of urea virtually throughout the incubation period, and (4) the late appearance of uricotelic devices, it would appear that the black snake embryo shows greater affinity with the turtles than with the birds. Although this conclusion might be anticipated on genetic grounds, the oft-repeated and unwarranted inference that the metabolism of saurian reptile embryos is identical with that of birds would appear to justify its experimental confirmation.

SUMMARY

1. Post-deposition growth of the black snake embryo is characterized by k values as follows: days 1–11, 0·46; days 11–34, 0·057; days 34–67, 0·039.

2. Total excreted nitrogen is 12·55 mg. occurring successively in development as ammonia, urea and uric acid; k value of total nitrogen production is 0·062, days 11–67, which in comparison with those of growth during this period suggest that stored protein is an energy source during a large part of development.

3. Urea is excreted into the albumen which is the principal storage reservoir and into the yolk; that which is excreted into the yolk is reabsorbed after the 45th day and re-deposited in the albumen as urea, and is in part (22%) converted to uric acid.

4. Concentration of urea in the yolk and yolk-sac continues to increase at the same rate after the 11th day ($k = 0{\cdot}024$), although the actual amount declines after the 45th day; concentration in the embryonic tissues increases to the 58th day, then decreases sharply to hatching time; concentration in the albumen increases throughout development, reaching a concentration of approximately 500 mg. %.

5. Uric acid synthesis is believed to be preceded by urea formation, and the presence of urease in the foetal liver and kidney suggest that the urea is hydrolysed to ammonia which is then incorporated into uric acid. Site of the transformation is uncertain, though the early and persistent localization of uric acid in the chorio-allantoic membrane points to this as the organ of synthesis. Decrease in total urea content is quantitatively identical with increase in uric acid.

6. The significance of these findings in relation to development of the vertebrate cleidoic egg is discussed.

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METABOLISM OF THE BLACK SNAKE EMBRYO

II. RESPIRATORY EXCHANGE

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(*Received* 6 *December* 1952)

Bohr (1904) reported CO_2 production of an embryo of *Natrix natrix*, weighing 0·46 g., to be 120 mm.^{3}/g./hr. at 14·2° C.; the CO_2 produced at 28° C. was 3 times that at 15° C., and production by a newly hatched snake at 15° C. was approximately 90 mm.^{3}/g./hr. Other data are given, but they are not related to stage of development, and oxygen utilization was not measured. Zarrow & Pomerat (1937) made rather exhaustive studies of the effect of temperature on various aspects of respiratory behaviour of the smooth green snake during the last week of development. It appeared profitable, therefore, to obtain details of respiratory exchange throughout the developmental period of snake embryos. Such data were available from the same embryos which served for the excretion studies reported elsewhere in this *Journal* by the author (1953).

MATERIALS AND METHODS

Data on oxygen consumption, CO_2 production and R.Q. were obtained by use of a special flask (Aminco 5-210) with a standard Warburg apparatus. Temperature was maintained at 23·9° C. during the determinations, although the environmental temperature between observation periods fluctuated between 21 and 30° C. The period of thermal adjustment of the egg prior to actual determinations was therefore various. CO_2 was absorbed by 20% KOH. After thermal equilibrium had been attained, the initial determination was made without KOH, giving a measurement of O_2–CO_2; KOH was then added to the flask, a second more brief period of thermal adjustment followed, and the determination of consumed O_2 was made. Subtraction of the former from the latter yields the value of CO_2 produced. The readings of CO_2 produced and O_2 consumed are therefore made within 1–3 hr., which interval in a developmental period of more than 2 months is not considered to be significant. The individual observations extended over periods of 1–3 hr. The values obtained were corrected for minor barometric and thermal fluctuations in the usual way.

RESULTS

Oxygen consumption. The relation of total consumed oxygen to growth of the embryo and its membranes is shown in Fig. 1. Parallelism between the embryonic growth and oxygen consumption is self-evident, but of particular note is the obvious divergence of the curves at the beginning of development. The explanation may lie partly in the fact that the first 3 weeks constitute the period of most rapid growth of the yolk-sac; it is also conceivable that during intra-uterine development an oxygen debt is created, which is repaid during the initial period of exposure to the atmosphere. Although there is a degree of increased vascularization of the uterus, the developing embryo is isolated from an oxygen source by its own shell and shell membrane, and must rely on diffusion rather than a special oxygen transport device for oxygenation of its tissues. The possibility of post-deposition oxidation of hydrogen acceptors in the yolk or elsewhere, or of other mode of oxidation, is not remote in light of the fact that newly laid infertile eggs, eggs from which only the embryo was removed, and isolated masses of yolk were observed to take up oxygen at the rate of 150 mm.^{3}/hr. (egg weight, $5{\cdot}0 \pm 0{\cdot}5$ g.). The question of bacterial contamination existed, and inoculation of a variety of media from several parts of eggs was carried out. These proved entirely negative. Such bacteria as were found were from

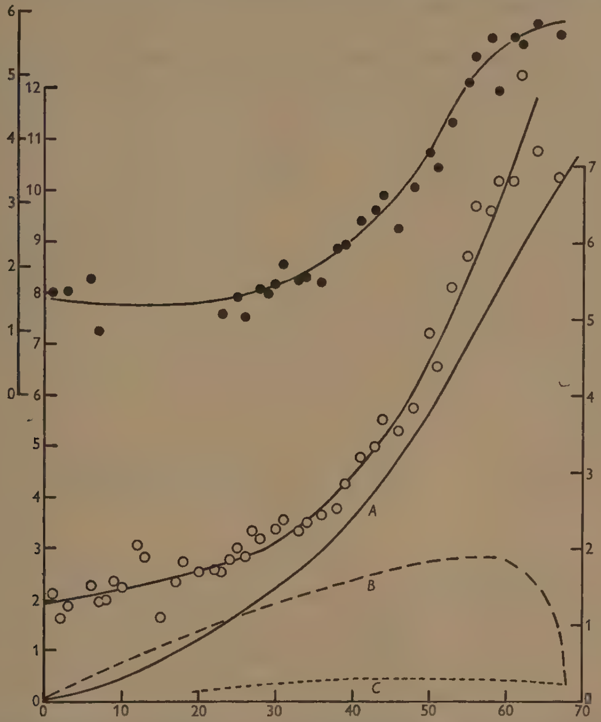

Fig. 1. Oxygen consumption (open circles) and carbon dioxide production (closed circles) throughout development. Smoothed growth curves of embryo, yolk-sac and chorio-allantoic membrane (*A*, *B* and *C*, respectively) are reproduced to make it easier to relate respiration to growth. Ordinate: right-hand scale, g.; left-hand scales, lower: oxygen consumption, mm.^{3}/hr. in hundreds; upper: CO_2 produced, mm.^{3}/hr. in hundreds. Abscissa: age in days after deposition.

the egg surface and were, of course, present on normal eggs. (The author is indebted to Dr R. A. Cleverdon for these findings.)

Further evidence of the high rate of initial metabolism of the intact egg is provided in Fig. 2. The metabolic rate calculated on the basis of embryonic weight alone, or combined embryonic and extra-embryonic tissues, during the first 5 days, is not greatly different from that of the chick, developing at a temperature of 39° C. Thereafter, however, the respiratory rate is much less than that of the chick (Beyer, 1952). The reference to combined weight of embryo plus membranes in the same figure presumes the same rate of metabolism for both, and in this respect may be somewhat misleading, since in fact no data are available on the separate rates of the embryonic and extra-embryonic tissues.

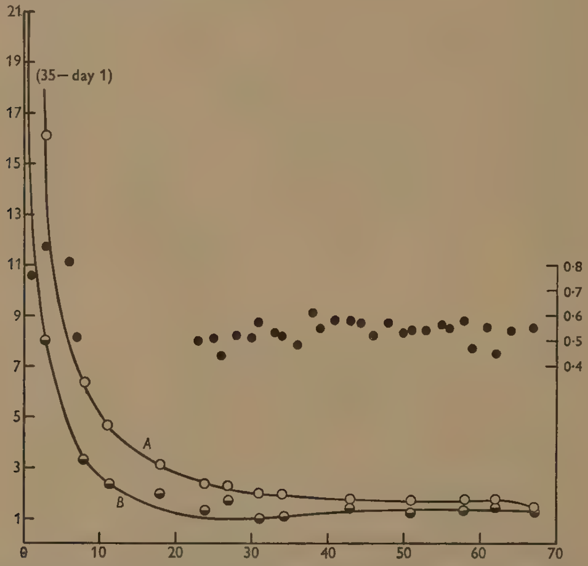

Fig. 2. Metabolic rate, based on embryonic weight (*A*) and combined weights of embryo and yolk-sac (*B*). Ordinate: mm.^{3} O_2/g./hr. in hundreds. Abscissa: age in days after deposition. Solid circles, R.Q. (ordinate on right-hand scale).

Carbon dioxide production. The curve of total CO_2 produced (Fig. 1) is shown in relation to O_2 consumed and embryonic growth. It is apparent that CO_2 production is initially relatively higher than the rate of O_2 consumption, and this is confirmed by the higher R.Q. values of days 1–8 (Fig. 2). This may reflect a utilization of carbohydrate as an energy source during the early part of development as suggested by Needham (1942). If supplemented by protein at this period, when ammonia is the end product, the R.Q. would also remain high.

Of particular interest is the persistently low R.Q. values from the 23rd day onward, having a mean value of 0·53. It was concluded from the study of excreted nitrogen that during the latter part of development protein (degraded to urea and later to uric acid) supplemented fat as an energy source. The maximal R.Q. which might be expected on the basis of combustion therefore would be approximately 0·7. The observed R.Q. may be accounted for by allocation to non-combustive processes of approximately 100 ml. of respiratory gases. The following possibilities suggest themselves: (1) ureotelic and uricotelic metabolism of protein, in which process potential CO_2 is incorporated in the excreted molecules; (2) oxygen used in pigment formation, which since it is consumed, increases the denominator of the fraction but does not lead to excretion of CO_2; (3) conversion of fat to carbohydrate, having a tendency to depress the R.Q. for the reason cited in item (2); and (4) the possible fixation of CO_2 in the form of organic acid or carbohydrate. Quantitative data are available only for the potential CO_2 of excretory products (approximately 5·0 ml.). Colorimetric estimation of liver glycogen of newly-hatched, juvenile and adult snakes by the method of Van der Kleij (1951) indicates that a substantially greater quantity is present in the newly hatched than in either of the two latter. Such data, though only suggestive, support the hypothesis of carbohydrate synthesis. It is hardly conceivable that more than 10% of the unaccounted gas can be concerned with pigment synthesis. The possibility of CO_2 fixation is supported by observations of several workers, of which Wood (1946) and Crane & Ball (1951) may be consulted for further references. Both found pigeon liver most effective, though other tissues also fixed CO_2; pyruvate is known to be involved, though the latter authors point out that there are other, unidentified avenues of incorporation of CO_2 in organic molecules.

These speculations do not constitute a solution to the problem, but possess the merit of amenability to experimental analysis.

SUMMARY

Data on exchange of respiratory gases are presented for the entire post-deposition period of development of the black snake. Of special note among the data are (1) an initial relatively high rate of O_2 consumption and CO_2 production, (2) an initially high R.Q., followed by a persistently low R.Q. (0·53).

Suggestions to account for the peculiarities are offered.

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CLEAVAGE OF SEA-URCHIN EGGS IN COLCHICINE

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(With Plates 12 and 13)

(*Received* 12 *December* 1952)

INTRODUCTION

We have recently put forward a theory linking mitosis with cleavage, in which we suggest that cleavage is caused by an expansion of the cell cortex which starts at either pole of the cell and later moves round to the region of the furrow (Mitchison, 1952). There is evidence that this expansion is brought about by substances diffusing from the two groups of daughter chromosomes which, by the end of anaphase, lie near the poles of the cell (Swann 1951 *a*, *b*). If this theory is correct, the important structure in mitosis is the spindle which is responsible for separating the two groups of chromosomes. The asters, where they exist, should act only as passive guides for the advancing furrow.

Some theories of cell division, on the other hand, e.g. Gray (1924), Dan (1943), suppose that the spindle and asters have an active function in cleavage, and that the cell surface is pulled passively into the furrow. It should be possible to distinguish between these opposing views by suppressing the spindle and asters at a point when the chromosomes have already separated, and seeing whether cleavage still continues. The most satisfactory way of doing this is to use colchicine. The action of this poison in stopping cleavage and suppressing the formation of the spindle without affecting the condensation of the chromosomes, has been known for some time, and there is a large literature on the subject (Eigsti, 1947). Its action on sea-urchin eggs has also been studied by a number of workers including Nebel (1937), Wilbur (1940) and Beams & Evans (1940). There is general agreement that colchicine, at concentrations of about 5×10^{-4} M inhibits mitosis and cleavage in these eggs. It prevents the formation of the spindle and asters, or, if they are already formed, it causes them to solate and disappear. The normal viscosity rise associated with the growth of fibrous material in the mitotic figures is therefore stopped, and viscosity remains low.

In most of the experiments, colchicine has been applied well before mitosis, making it impossible to separate its effects on cleavage from its effects on mitosis. Beams & Evans (1940), however, made the significant observation that the critical point in mitosis for colchicine inhibition is about anaphase. If eggs are treated with colchicine before anaphase, cleavage is stopped, but if they are treated during or after anaphase, cleavage takes place, even though the asters appear, by ordinary light, to have vanished in both fresh and stained material. This suggests rather

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strongly that the spindle and asters are not required for cleavage. On the other hand, it is difficult to be certain by ordinary observation that the asters have actually disappeared at the moment of cleavage. At this time they are no longer very obviously fibrillar in ordinary light, and it would not be impossible to suppose that they had disappeared, when in fact they were still having a mechanical effect on the cleavage process.

There are two ways of confirming the disappearance of the asters. First, the eggs can be observed in polarized light. This is a very sensitive method of detecting the presence of oriented structures, and in a normal division the asters are in fact readily visible throughout cleavage and for a considerable time afterwards. We have therefore applied colchicine to sea-urchin eggs at different points in mitosis, and observed the effects under polarized light, using time-lapse photography. Secondly, there should be a fall in the viscosity of the egg contents associated with the disappearance of the asters. We have therefore measured the viscosity of cleaving *Arbacia* eggs by stratifying the pigment granules with a centrifuge after treatment with colchicine.

We have also attempted to fix more accurately the critical point in mitosis for colchicine inhibition by applying colchicine at different times and relating chromosome separation measured on stained material with the percentage cleavage.

It is known that cycles of nuclear activity, corresponding to the condensation of the chromosomes at mitosis, continue in many cells after treatment with colchicine, even though mitosis and cleavage have been inhibited (Nebel, 1937). We have found a similar continuance of cycles in sea-urchin eggs (cf. Zeuthen, 1951). On our theory this cycle should be paralleled by changes in the state of the cell cortex similar to those which take place in normal division; Monroy & Montalenti (1947) have, in fact, found such changes in the cortical birefringence and plasmolysis. We have followed this up by investigating cycles of change in the rigidity and light-scattering of the cortex.

EFFECT OF STRONG COLCHICINE ON CLEAVAGE

(*a*) *Observations in polarized light*

Although colchicine at 5×10^{-4} M suppresses the spindle and asters, it only does so rather slowly, and the last traces of birefringence do not disappear for 10–15 min. With stronger concentrations, however, the birefringence vanishes more rapidly. In the experiments described below, colchicine was used at $3{\cdot}2 \times 10^{-3}$ M; under these conditions the birefringence disappears completely in 2–3 min.* In all the experiments, the fertilization membranes were removed, and the eggs (*Psammechinus miliaris*) put in calcium-free sea water to dissolve away the hyaline layer. Room temperature was about 18° C.

The normal appearance of mitosis and cleavage in polarized light is shown in Pl. 12, figs. 1–8. The interpretation of these birefringence changes has been dealt with in some detail by Swann (1951 *a*, *b*). It is only necessary to say here that in metaphase the spindle and asters are small and brightly birefringent, while during anaphase they get steadily larger and fainter. The centrosomes also move apart. At the end of anaphase the spindle is no longer visible as a separate structure, while the asters become very large and virtually fill the cell but are considerably fainter than at metaphase. They continue in this condition throughout cleavage. With a little experience it is possible to tell the particular stage of the eggs with some accuracy from their appearance in polarized light.

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* Inoué (1952) finds that this concentration of colchicine causes the recession of the spindle in *Chaetopterus* eggs in 4 min.

The effect of colchicine is shown in Pl. 12, figs. 9–12, and Pl. 13, figs. 1–16. In each case photography was begun 1 min. after the eggs were put in colchicine, the subsequent photographs being selected at 2 min. intervals. In Pl. 12, figs. 9–12, the eggs were put into colchicine during metaphase. The spindle and asters disappeared rapidly, and there were no signs of cleavage. In Pl. 13, figs. 1–8, on the other hand, the eggs were not put into colchicine until anaphase was more or less complete. Once again the asters disappeared rapidly, but cleavage continued with no trace of birefringence in the cells. The most interesting case is shown in Pl. 13, figs. 9–16. Here the colchicine was put on when the eggs were in anaphase. The two lower eggs were in mid-anaphase (shown by relatively large and fairly widely spaced asters of medium brightness) and presumably developed a little further before they were arrested. Both these eggs completed cleavage, though rather slowly. The centre and top left eggs, on the other hand, were caught quite early in anaphase and presumably got no further than mid-anaphase. They do, however, show elongation and the beginning of a furrow, though this ultimately retracts. The top right egg was caught in metaphase and showed no signs of cleavage at all.

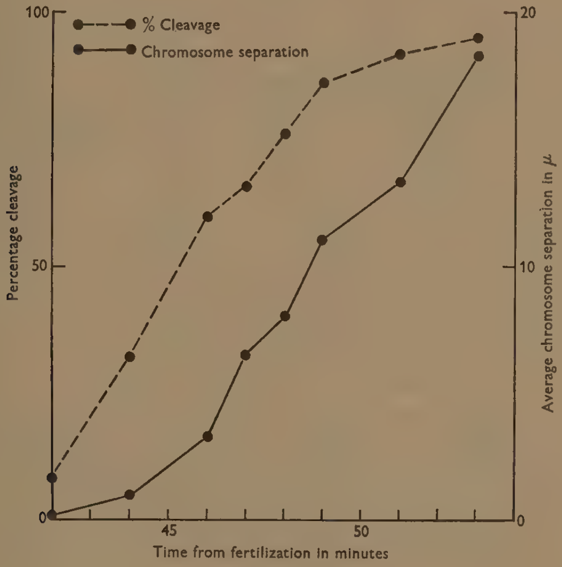
An interesting feature of eggs dividing in colchicine, also described by Beams & Evans (1940), is the irregular cleavage shapes which they often show. This can be seen to some extent in Pl. 13, figs. 3 and 13, but it is much more striking in the four selected eggs (in ordinary light) shown in Pl. 12, figs. 13–16. A common feature in these irregular cleavages is for the two sides of the furrow not to lie opposite each other.

(*b*) *Observations on stained sections*

Eggs of *P. miliaris* were put into 3×10^{-3} M colchicine at various times in anaphase. At the same times, small batches of eggs were fixed. The colchicine-treated eggs were later counted for percentage cleavage, and the fixed eggs were sectioned and stained, and measured for chromosome separation. Values for percentage cleavage (based on 200 eggs) and for average chromosome separation (based on fifty measurements) are plotted together, against time after fertilization (at 18° C.), in Text-fig. 1. Ideally the curve for percentage cleavage should show a sudden transition from 0 to 100%. This is not, of course, realized in practice, but it will be seen that there is a fairly sharp rise between 42 and 49 min. At these times the average chromosome separation is between 0 and 11 μ. It is evident, therefore, that a chromosome separation of the order of 5 μ is necessary for cleavage to take place.

(*c*) *Viscosity changes*

The effect of colchicine on cytoplasmic viscosity at cleavage was examined in the eggs of *Arbacia lixula*, using the criterion of stratification of the pigment granules with a centrifuge as a measure of the internal viscosity. At 45 min. after fertilization (at 29° C.) the first few eggs of a batch were starting to cleave, so the majority of the batch could be assumed to be in anaphase. The batch was then divided into two lots, one lot being mixed with colchicine in 0·95 M-sucrose to give a solution of the same density as the eggs and with a concentration of colchicine of 3×10^{-3} M, while the other lot was mixed with sucrose alone as a control. Samples were then taken from the control eggs, and from the eggs in colchicine after 3, 8 and 11 min. from mixing, and centrifuged at 25,000 g. for 3 min. This speed and time of centrifuging was sufficient to give sharp stratification of the pigment granules in unfertilized eggs, but no stratification in fertilized ones. At the end of the centrifuging each sample was fixed in formalin, and eventually counted in order to find the percentage cleaved or cleaving, and the percentage showing sharp stratification. The results of an experiment are given in Table 1.



Text-fig. 1.

These results show a definite rise in the number of stratified cleaving eggs from 5·5% in the controls to 51% after 3 min. in colchicine and 72·5% after 8 min. This shows that colchicine reduced the viscosity of most of the cleaving eggs to a value about as low as that in the unfertilized egg. This is presumably due to the disappearance of the asters. The results are not as clear-cut as might perhaps be expected: ideally the percentage of stratified eggs should rise from 0 to 100. However, the initial value of 5·5% in the control is probably due to the presence of a few unfertilized or cytolytic eggs, and the final values of 51 and 72·5% may be underestimates since a stratified egg viewed from either end (rather than from the side) may appear to be unstratified. The drop in the percentage of cleaving eggs from sample B to sample C is presumably due to the recession of the furrow which takes place in the more retarded eggs of a batch.

Table 1

Sample	Time in min. from addition of colchicine to start of centrifuging	Cleaving and cleaved eggs		Eggs not cleaved	
		% in sample	% stratified	% in sample	% stratified
Control	3 (No colch.)	72·5	5·5	27·5	5·9
A	3	53	51	47	51
B	8	63	72·5	37	65
C	11	47·5	71	52·5	64

Final cleavage in controls, 92%.

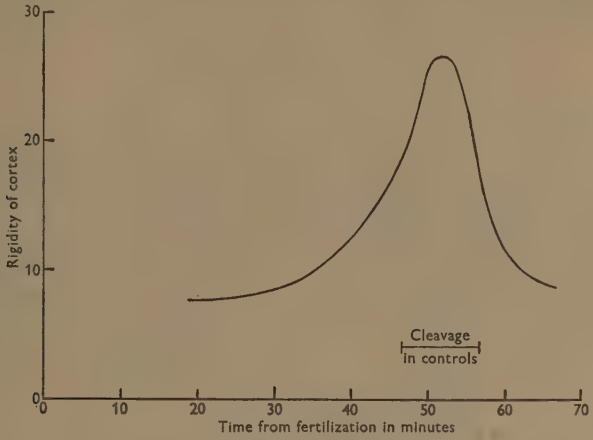
CYCLICAL CHANGES OF CORTEX AND CHROMOSOMES IN WEAK COLCHICINE

(a) *Changes in the rigidity of the cortex*

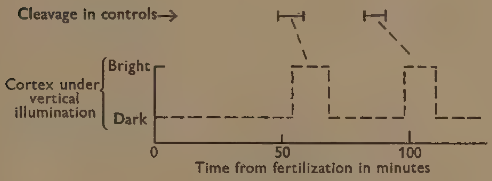
We have devised a method of measuring the rigidity of the cortex by using what we have called a 'cell elastimeter' (Mitchison & Swann, 1954). This method consists essentially of sucking a small bulge from the egg surface into a micro-pipette. The size of the bulge for a given suction is a measure of the rigidity of the cortex. In the normal developing egg, the rigidity is low in interphase, rises at cleavage, and then falls again in the next interphase. This cycle persists in colchicine-treated eggs, even though cleavage is inhibited. Text-fig. 2 shows the change of rigidity with time for eggs of *Psammechinus militaris* placed in 2×10^{-4} M-colchicine in calcium-free sea water 15 min. after fertilization (at 18° C.). The rigidity is given in arbitrary units which are roughly proportional to the elastic modulus of the cortex. The period of cleavage in untreated controls is also shown. The rise in rigidity at a time roughly corresponding to the time of cleavage in the controls is very evident, but it should be noted that this rise is only about one-sixth as great as in normal cleaving eggs.

(b) *Changes in the light-scattering properties of the cortex*

We have described elsewhere (Mitchison & Swann, 1952) the changes in the light-scattering properties of the cortex that take place during normal division. Under vertical illumination, the brightness of the cortex increases sharply at the start of anaphase and then diminishes during cleavage. The same cycle occurs in colchicine-treated eggs, though it is somewhat delayed. Text-fig. 3 shows the changes in cortical brightness with time for eggs of *P. miliaris* treated with 2×10^{-4} M-colchicine at 15 min. after fertilization (at 18° C.). The periods of cleavage in untreated controls are also shown.



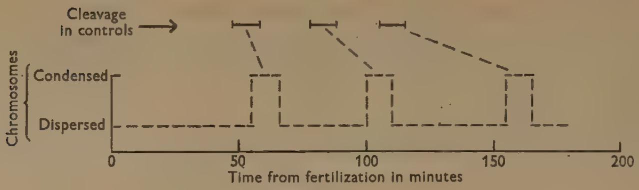
Text-fig. 2.



Text-fig. 3.

(*c*) *Changes in the chromosomes*

In order to follow the changes in the condensation of the chromosomes, a series of stained sections were made of colchicine-treated eggs of *P. miliaris*. The eggs were placed in 2×10^{-4} M-colchicine at 10 min. after fertilization (at 18° C.), and samples were then taken and fixed every 15 min. for 3 hr. The results are shown in Text-fig. 4, together with the cleavage periods in an untreated control. It can be seen that there are cyclical changes in the condensation of the chromosomes (which correspond to the changes in normal division), but these changes are somewhat delayed relative to the control, as in the previous experiment. This result is similar to that found by Zeuthen (1951) with *P. microtuberculatus*.



Text-fig. 4.

DISCUSSION

It is clear from these experiments that neither the spindle nor the asters are necessary for cleavage. The original observation of Beams & Evans (1940) has been confirmed, both with stained sections in ordinary light, and with the more sensitive method of polarized light. These observations show that colchicine destroys both the visible fibrous structure of an aster and its submicroscopic orientation, while the centrifuging experiments show that its gel structure is also destroyed. This is not to say that the asters normally play no part in division. Presumably they act as passive guides for the furrow, and their absence in colchicine-treated dividing eggs may well be responsible for the irregular cleavage shapes.*

Although colchicine is probably the most convenient way of destroying the asters in a dividing cell, other methods have been used and have yielded similar results. Chambers (1938) destroyed the asters in a dividing egg by stirring with a microneedle, and found that cleavage continued. We have repeated and confirmed this observation (Mitchison, 1953). Moreover, Tahmisian (in discussion in Inoué, 1952) states that cells will divide when their asters have been destroyed by irradiation.

These observations, we believe, destroy any theory of cell division which involves the spindle and asters as necessary agents in the cleavage of the cytoplasm (e.g. Gray, 1924; Dan, 1943). They are, however, quite consistent with the 'expanding membrane' theory of cell division we have put forward (Mitchison, 1952), where the active mechanical agent in cleavage is the cell cortex, controlled by the diffusion of substances from the two groups of daughter chromosomes. We should expect on this theory that cleavage should take place if the chromosome groups are well separated, despite the destruction of the asters by colchicine. If, however, the chromosomes are prevented from separating by the destruction of the spindle, cleavage should be stopped. This, in fact, is what appears to happen. Beams & Evans showed that the critical point in mitosis for colchicine inhibition is about anaphase; we have found that there must be a separation of about $5\,\mu$ between the chromosome groups for cleavage to occur in colchicine.

Since changes in the optical and mechanical properties of the cortex are normally associated with the condensation of the chromosomes in mitosis, we should also expect that similar changes should be associated with chromosome condensation in colchicine-treated eggs, even though the destruction of the spindle has prevented chromosome separation, and therefore cleavage (Swann, 1952). These changes have, in fact, been shown to take place both in birefringence and plasmolysis by Monroy & Montalenti (1947), and in light-scattering and rigidity in the experiments described in this paper.

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* Similar shapes occur in cleaving eggs whose asters have been destroyed by stirring with a microneedle (Mitchison, 1953).

SUMMARY

1. Sea-urchin eggs have been treated with strong colchicine during mitosis, and examined with a variety of techniques.

2. Polarized light observations show that cleavage can take place when the asters and spindle have completely disappeared, provided the cell has reached mid-anaphase, by which time the chromosome groups are well separated.

3. Observations on stained sections have confirmed those with polarized light and have also shown that the minimum separation of the chromosome groups for successful cleavage is about 5 μ.

4. Viscosity measurements have shown that colchicine destroys the gelated structure of the asters, and reduces the viscosity of the cleaving egg to about the same low value as the unfertilized egg.

5. Cycles of chromosome condensation persist in eggs treated with weak colchicine, even though cleavage has been prevented by an early application of the colchicine. These cycles are associated with changes in the rigidity and light-scattering properties of the cortex.

6. The relevance of these observations to some theories of cell division is discussed.

We wish to thank the Director and Staff of the Marine Station, Millport, and of the Stazione Zoologica, Naples, for their help in this work.

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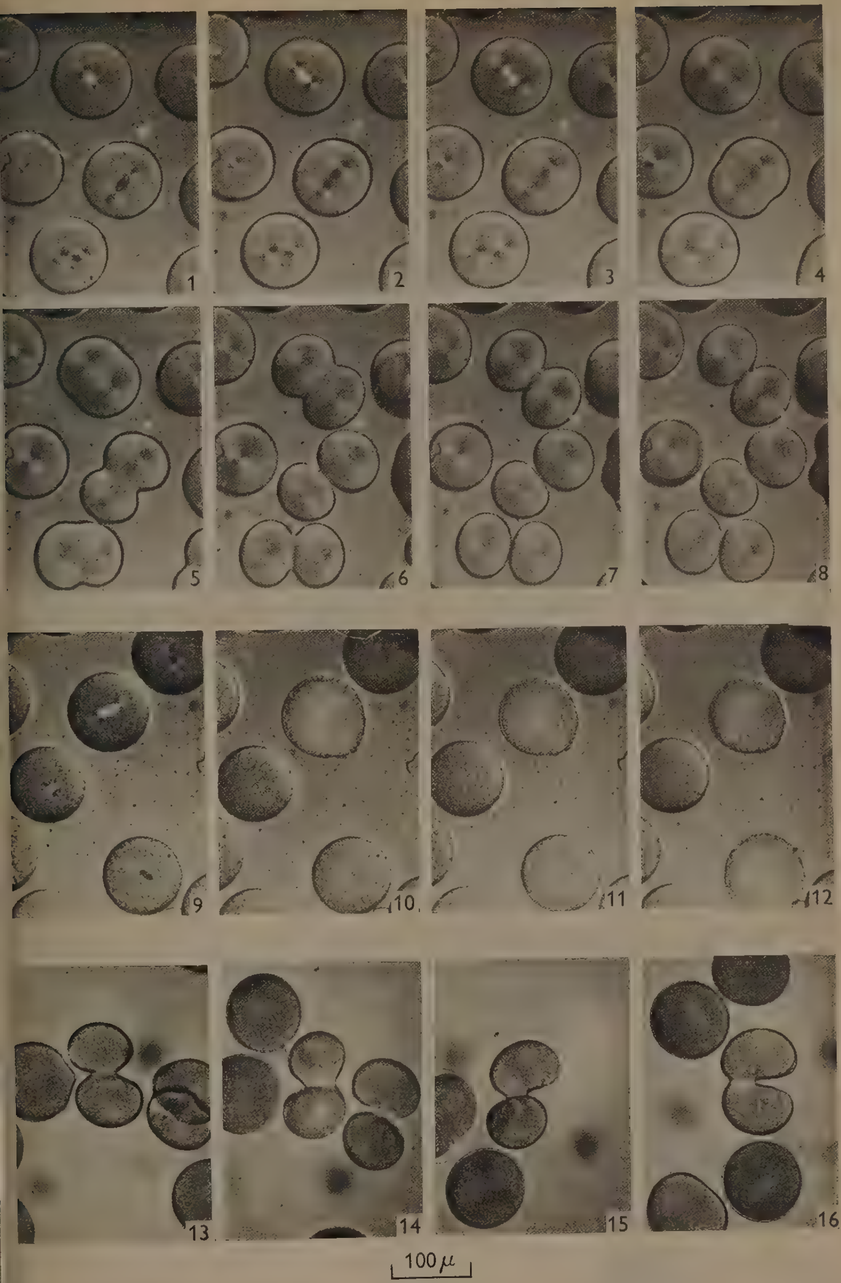
EXPLANATION OF PLATES 12 AND 13

PLATE 12

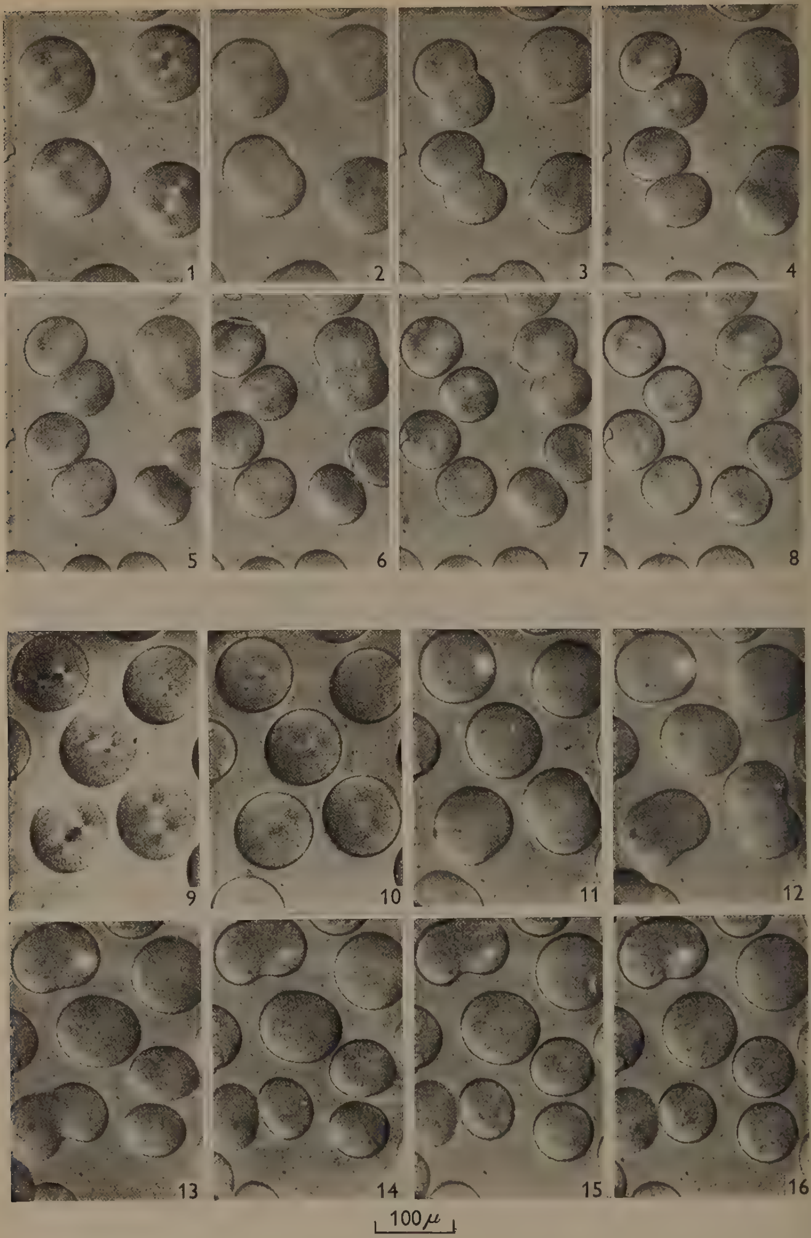
Figs. 1–16. *Psammechinus miliaris* eggs. Figs. 1–8. Normal cleavage. 2 min. intervals. Polarized light, with compensation. Figs. 9–12. After treatment with colchicine at metaphase. 2 min. intervals. Polarized light, with compensation. Figs. 13–16. Irregular cleavage shapes after treatment with colchicine at anaphase. Ordinary light.

PLATE 13

Figs. 1–16. *Psammechinus miliaris* eggs. 2 min. intervals. Polarized light, with compensation. Figs. 1–8. After treatment with colchicine at telophase. Figs. 9–16. After treatment with colchicine at anaphase.



SWANN AND MITCHISON—CLEAVAGE OF SEA-URCHIN EGGS IN COLCHICINE



SWANN AND MITCHISON—CLEAVAGE OF SEA-URCHIN EGGS IN COLCHICINE

MICRODISSECTION EXPERIMENTS ON SEA-URCHIN EGGS AT CLEAVAGE

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(With Plates 14 and 15)

(*Received 20 January* 1953)

INTRODUCTION

This paper describes a series of microdissection experiments on sea-urchin eggs which were done in the course of investigations into the mechanism of cell division. It was hoped that these experiments would throw some light on the validity of a theory of cleavage which M. M. Swann and I have developed and which we have called the 'expanding membrane' theory. This theory has been outlined earlier (Mitchison, 1952), and it is only necessary here to describe a few of the salient points which are relevant to the microdissection experiments. We believe that cleavage is started by the diffusion of a substance from the two groups of daughter chromosomes at the end of anaphase. This substance reaches the poles of the cell and starts a wave of expansion in the cell membrane or cortex which gradually spreads round to the equator. The furrow is formed initially by the passive contraction of the elastic cortex at the equator, but its subsequent advance is due to an active inwards expansion of the cortical material in the furrow wall when the diffusing substance reaches it. In order that this mechanism should work, there must be no tension in the cell membrane during the later stages of cleavage and therefore no internal pressure in the cell. The asters are not thought to play an active part in the process, but only to act as passive guides for the advancing furrow and thus ensure an orderly cleavage.

There are two other main theories of cleavage. In one, cleavage is thought to be due to the contraction of a ring of gelated material at the equator, e.g. Marsland (1951). In the other, an astral mechanism is invoked, with the active process being either an expansion of the spindle remnant (Dan, 1943), or a growth of the asters (Gray, 1924).

Some of the experiments to be described are not original and were done for confirmation and, to some extent, clarification of results of Chambers, who has done the pioneer work in this field. Some of the experiments on tearing blastomeres have already been described in Mitchison (1952).

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METHOD

The sea-urchin eggs used were those of *Psammechinus miliaris*, except in the experiments on tearing blastomeres where the eggs of *Arbacia lixula* were also used. It was necessary to remove the fertilization membrane and any traces of the egg jelly. This could be done quite easily with the *Psammechinus* eggs at Millport by passing them through fine mesh bolting silk about 2 min. after fertilization. In the case of the *Arbacia* eggs at Naples, the procedure was more complicated and produced a larger number of broken eggs. The jelly was removed by treating the unfertilized eggs for a short time with acidified sea water. The fertilization membranes were removed by placing the eggs in isotonic urea about 2 min. after fertilization, stripping off the swollen membranes by passing through bolting silk, and then washing off the urea as soon as possible.

The calcium-free (Ca-free) sea water used to remove the hyaline layer in some of the experiments was made up of NaCl, 60 pts.; $MgSO_4$, 13; KCl, 1·5; $NaHCO_3$, 1; adjusted to isotonicity with distilled water. The calcium and magnesium-free (Ca and Mg-free) sea water used in the blastomere puncturing experiment was made up of NaCl, 65 pts.; KCl 1·75; $NaHCO_3$, 1; adjusted to isotonicity.

All the experiments were done with a Zeiss Peterfi micromanipulator under a 16 mm. objective, and the microdissection instruments were made with a de Fonbrune microforge. Room temperatures varied from 17 to 21° C.

TEARING ONE BLASTOMERE OF A CLEAVING EGG

Chambers (1938) described a microdissection experiment in which he tore open one blastomere at a dumb-bell stage in *Arbacia punctulata* eggs which were cleaving in KCl isotonic with sea water. The damaged blastomere disintegrated, and he said that 'the absence of divalent cations in the medium permitted the continued outflow...of the interior of the egg...'. If the cleavage furrow was far advanced, it continued its course after the operation, and closed off the intact blastomere. If, on the other hand, it was not far advanced '...the contents of the untorn blastomere flowed through the connecting stalk while the blastomere shrank in size much like a gradually deflating balloon'. In this case both the intact and the torn blastomere disintegrated. Sichel & Burton (1936) assumed from this experiment that there was a positive tension in the membrane, and calculated it to be 0·09 dyne/cm. It is an essential point in the 'expanding membrane' theory that there should not be a positive tension in the intact membrane during the later stages of cleavage, so it was decided to repeat these experiments and see whether the interpretation seemed correct. It also seemed necessary to try this experiment in other media since Chambers appeared to have found differing results in them. In his paper of 1938, he says that the major part of one of the blastomeres of a cleaving egg can be cut away without disturbing the course of the cleavage furrow, and that the presence of the hyaline layer is not essential. This would imply that the deflating balloon effect does not occur either in ordinary or in Ca-free sea water. On the other hand, he describes later (Chambers, 1951) the deflating balloon experiment as being done in Ca-free sea water. In another paper (Chambers, Chambers & Leonard, 1949), he describes a contraction of the egg and an outflow of the contents when punctured at the astral stage in a medium containing 9 parts NaCl to 1 part KCl. This medium is free of calcium and magnesium, but it has over four times as much potassium as is normally present in sea water.

Preliminary experiments were done on *Psammechinus miliaris* eggs in ordinary, and in Ca-free sea water. When the dividing eggs had reached the dumb-bell stage, they were gripped over the furrow by a holding needle (shaped like a question mark), and one of the blastomeres was torn in two by a slicing cut with a straight needle. In ordinary sea water, the cut sealed at once and the egg continued to cleave with the torn blastomere represented by a small and usually irregular shaped fragment. In Ca-free sea water, the reaction which followed the puncturing depended on the diameter of the furrow neck. If the furrow was less than about 30μ, the furrow continued to advance and sealed off the intact blastomere (Pl. 14, figs. 1, 2). There was no flow through the neck, and the intact blastomere was unaffected. The torn blastomere, however, showed a striking injury reaction which spread through it in about 10 sec. and which appeared as a granulation and slight swelling of cytoplasm, a roughening of the surface, and the assumption of a brownish colour in transmitted light (this is probably the same as the 'black cytolysis' of some authors). The torn blastomere did not dissolve away. If, on the other hand, the furrow diameter was greater than about 30μ, the injury reaction spread through the furrow neck to the intact blastomere which swelled a little and turned the same brownish colour as the torn blastomere (Pl. 14, figs. 3–5). This was accompanied by a partial retraction of the furrow and by a momentary movement of the cytoplasm in the furrow neck towards the torn blastomere, but these reactions appeared to be due rather to the swelling of the cytoplasm in the previously intact blastomere than to the contraction of its membrane. There were no signs of the regular continuous movement, or the contraction of the previously intact blastomere, which were found by Chambers.

The main experiments were done in a number of different media on the eggs of *Arbacia lixula*, since this sea urchin is the nearest equivalent in Europe to the American *A. punctulata* used by Chambers. In ordinary sea water, in Ca-free sea water, and in Ca and Mg-free sea water, the *Arbacia* eggs reacted to tearing in the same way as the *Psammechinus* eggs in ordinary sea water. It was impossible to cause cytolysis or outflow from the eggs since the cuts sealed up at once, and cleavage continued with large parts of one blastomere removed. This is shown in Pl. 14, figs. 6–10; fig. 6 shows a cleaving egg in Ca-free sea water under the holding needle; fig. 7 shows it after a part of one blastomere has been removed by a cut, and figs. 8 and 9 show the completion of cleavage; fig. 10 is another egg which has nearly completed cleavage with only a very small part left of the torn blastomere. These photographs emphasize two points which are relevant to theories of cleavage. First, that cleavage can continue with one blastomere in an irregular shape. If there was any substantial internal pressure in the cleaving egg, this blastomere should round up at once. Secondly, the fact that nearly all of one blastomere, together with its astral apparatus, can be removed without stopping cleavage, argues against an astral mechanism of cell division.

If *Arbacia* eggs were allowed to develop after fertilization in either ordinary or Ca-free sea water and then placed in isotonic KCl when they were cleaving, they reacted to cutting in exactly the same way as the *Psammechinus* eggs in Ca-free sea water. If the furrow was nearly closed, only the torn blastomere cytolysed, while if the furrow had only advanced half way through or less both blastomeres cytolysed (Pl. 14, fig. 11). There was no outflow and no contraction of the previously intact blastomere. The only difference between *Arbacia* and *Psammechinus* was that in the former the injury reaction was less conspicuous because of the dark colour of the eggs. The conditions of this experiment are as nearly as possible identical with those in the deflating balloon experiment of Chambers, so the finding of a different result seems to be due to the use of another species of *Arbacia*.

It is important at this point to stress the fact that isotonic KCl is a most unsatisfactory medium to use for microdissection experiments on developing eggs. The most obvious objection to it is that *A. lixula* eggs would neither develop nor cleave in it unless they were placed there while they were actually cleaving. Even then, only those eggs which were already in the dumb-bell stage completed cleavage, though very slowly and often irregularly. Eggs which were in earlier stages reverted back either to spheres or, more often, to irregular shapes. There was often a curious stratification of the egg contents so that one end of an egg appeared much redder than the other. These changes are shown in Pl. 14, fig. 12, which is a photograph of a field of *Arbacia* eggs placed in isotonic KCl at cleavage. It should be added that eggs develop and cleave normally both in Ca-free and in Ca and Mg-free sea water.

It is apparent from these experiments that the result of puncturing one blastomere of a cleaving sea-urchin egg depends to some extent on the species of sea urchin. The eggs of *Psammechinus miliaris* are unaffected in ordinary sea water but cytolyse in Ca-free sea water. The eggs of *Arbacia lixula* are more resistant; they are unaffected in ordinary, Ca-free and Ca and Mg-free sea water, and only cytolyse in KCl. The main difference between the eggs of *Arbacia lixula* and those of *A. punctulata* appears to be that the latter show the deflating balloon effect in KCl while the former do not.

There are, however, definite points of similarity between the reactions of these eggs. In the first place, *Psammechinus* and *Arbacia lixula* show the same kind of cytolysis. Secondly, it appears that the two species of *Arbacia* only cytolyse in abnormally high concentrations of KCl. This statement implies the following interpretation of Chambers's work on *A. punctulata*: that cleaving eggs cytolyse in isotonic KCl but not in ordinary or Ca-free sea water (Chambers 1938); that the cytolysis in the mixture of 9 parts NaCl and 1 part KCl (Chambers *et al.*, 1949) is due to the high proportion of KCl; and that when Chambers (1951) describes the deflating balloon effect in Ca-free sea water he is, in fact, describing his original experiment which was done in isotonic KCl. Thirdly, although the deflating balloon effect and eventual dissolution occur only in *A. punctulata*, the cytolytic breakdowns in all three eggs show certain common features. There is an injury reaction which spreads over the egg (this reaction is described by Chambers in his paper of 1951, though not in his paper of 1938), this reaction spreads to the intact blastomere only if the furrow neck is still comparatively large, and there is flow or indications of movement in the furrow neck only when the injury reaction has spread to the intact blastomere and it is in the process of cytolysing.

In view of the fact that *A. lixula* eggs are unaffected by cutting in Ca and Mg-free sea water, it seems more probable that the cytolysis and continued outflow of *A. punctulata* eggs in isotonic KCl is due to the high concentration of K^+ ions rather than to the absence of divalent ions as suggested by Chambers.

In summary, it seems that no legitimate conclusions about the normal state of the cell membrane can be made from the deflating balloon effect for the following reasons: (1) the effect in *A. punctulata* only occurs in isotonic KCl (or, possibly, in abnormally high concentrations of K^+ ions). In the nearly related species *A. lixula* the effect does not occur, and, in any case, isotonic KCl appears to be a very unsatisfactory medium since it inhibits division unless the eggs are placed in it during the actual process of cleavage. (2) The effect only occurs after an injury reaction has spread through the intact blastomere and while the whole egg is in the process of cytolysis.

We can conclude, therefore, that Sichel & Burton's deduction of a positive membrane tension apply only to one species of sea-urchin egg, in an abnormal medium and after it has suffered irreparable damage. The fact that eggs will continue to cleave with one blastomere in an irregular shape indicates that, on the contrary, there is no membrane tension and no internal pressure. These are the conditions demanded by the expanding membrane theory of cleavage.

PASSAGE OF THE CLEAVAGE FURROW THROUGH A NEEDLE

These experiments were done in order to see whether the cleavage furrow could pass through an obstruction placed in its path—in this case, a microneedle. A cleaving egg in ordinary sea water was held between two microloops (about 50 μ diameter, and placed vertically) and a microneedle was then inserted into the egg and through the loops. The needle was inserted off the central (polar) axis of the egg and was either parallel to the central axis, i.e. paraxial (Pl. 14, figs. 13, 14), or parallel to the equator and piercing the bottom of the furrow at two places (Pl. 14, figs. 15–17). In the first case the advancing furrow passed through the needle without any tendency to gape and eventually left the needle transfixing the two blastomeres. In the second case the furrow also passed through the needle but left it free of the egg.

These results do not appear to be consistent with the idea of a simple contracting ring at the bottom of the furrow being responsible for cleavage. If there was such a ring, it is most improbable that it could pass through a paraxial needle without a serious distortion. Its continuity would be broken, and, since it must be under tension, it should gape. A more likely effect with a contracting ring would be that as soon as the ring reached the needle it would tend to displace the egg on the needle so that the final effect would be the needle running along the central axis of the egg with the nearly complete furrow disposed symmetrically round it.

These difficulties do not arise with the expanding membrane theory since the furrow is being pushed inwards by the expansion of its own walls and the rest of the cell membrane, rather than being pulled inwards by a contracting ring. As a result a wound in the furrow would tend to close rather than to gape, and, since the motive force is provided by the expansion of a large area of membrane, a small break in it should have little effect.

CLEAVAGE WITHOUT ASTERS

Chambers (1938) made the important observation that if the asters in a cleaving egg were destroyed by stirring with a microneedle, cleavage nevertheless continued (though if this was done at an early amphiaster stage, cleavage was inhibited). Since this is a crucial piece of evidence against any astral mechanism of cleavage, it was decided to repeat this experiment.

The same instruments were used in this experiment as in the previous one, i.e. two microloops for holding the egg, and a microneedle. When the needle had been inserted into the egg (in ordinary sea water), it was moved round so that the contents of the egg were stirred up. The asters were initially distorted and then, after about a quarter of a minute, they vanished. If this was done in metaphase or early anaphase the eggs did not cleave, but if it was done when the egg had already started to change its shape preparatory to division, cleavage continued. This is shown in Pl. 15, figs 1–5: fig. 1 is the egg in the 'wall-sided' stage just before the operation; fig. 2 shows the egg being stirred; figs. 3–5 show the egg completing cleavage. Unfortunately, the photographs are not good enough to show the presence or absence of the asters, but in the experiment the asters had vanished in the stirred egg by the time fig. 3 was taken, whereas at this stage in a normal egg the asters could still be seen.

This experiment entirely confirms the results of Chambers and was done with a different sea urchin (*Psammechinus miliaris* rather than *Arbacia punctulata*). An independent confirmation of the fact that cleavage can take place without asters has also been provided by a study of the effects of colchicine on sea-urchin eggs (Swann & Mitchison, 1953).

There is one further point about this experiment that is worth making. The expanding membrane theory suggests that although the asters are not essential for cleavage, they do act as passive guides for the advancing furrow. One would therefore expect that the cleavage shapes should become somewhat irregular if the asters were destroyed. This, in fact, occurred in the stirring experiments; a common feature being the fact that the two sides of the furrow did not lie opposite each other (Pl. 15, fig. 3). An exactly similar appearance was shown by a number of cleaving eggs in which the asters had been destroyed by the action of colchicine (Swann & Mitchison, 1953).

COMPRESSING EGGS AT CLEAVAGE

A number of experiments were carried out on the effect of compression on cleaving eggs in order to see how far the results might be consistent with the various theories of cleavage. A certain amount of work has already been published on the effect of compressing sea-urchin eggs. Zeuthen (1951) compressed *Psammechinus* eggs under a cover-slip until their thickness was about 20 μ (one-fifth of their original diameter) and found that this inhibited cleavage although nuclear cycles continued. Danielli (1952) obtained similar results, though he states that the eggs would eventually divide if they were not too much compressed. Chambers *et al.* (1949) also compressed *Arbacia* eggs under a cover-slip fixed to a microneedle until their diameter was 2–3 times their original diameter and it appears from their paper that the eggs did not cleave. This experiment, however, was carried out in the high K^+ mixture mentioned earlier (p. 517). The general conclusion from this earlier work seems to be that cleavage is only inhibited if there is large amount of flattening.

The compression experiments were done on cleaving eggs in ordinary and in Ca-free sea water. The two microloops mentioned earlier were used to flatten the eggs, and the flattening was carried out both parallel to and at right angles to the polar axis.

If the eggs were flattened at right angles to the polar axis in ordinary sea water cleavage continued normally (Pl. 15, fig. 6). In Ca-free sea water, however, although cleavage continued it was somewhat delayed, in some cases up to 5 min. This is shown in Pl. 15, figs. 7 and 8. In fig. 7 the 'wall-sided' egg on the left is at an earlier stage of cleavage than the egg about to be compressed, while in fig. 8, 5 min. later, it has completed cleavage though the compressed egg has its furrow only half way through. It can be seen from these photographs that the difficulty of compressing eggs in this direction was that as soon as the furrow had formed, the loops tended to slip on to one blastomere. The resulting flattening was usually less than when the eggs were compressed along the polar axis, and the flattening was only effective on one blastomere.

If the eggs were flattened along the polar axis in ordinary sea water, cleavage continued but was delayed up to 5 min. compared to the surrounding eggs. In some cases the furrow reverted back before finally completing the cleavage. This is shown in Pl. 15, figs. 9–13. One side of the furrow has reverted in fig. 11. The other egg in the field started by being at the same stage in cleavage as the compressed egg (fig. 9), but completed cleavage when the compressed egg had its furrow only about one third through (fig. 12).

If the eggs were flattened along the polar axis in Ca-free sea water, cleavage was inhibited in all cases, unless the furrow was nearly complete. The furrow might show several attempts to advance but it finally reverted back. This is shown in Pl. 2, figs. 14–18. In this case the furrow reverted back twice. There was no sign of the coalescence between the two walls of the furrow which has been described by Chambers (1946); the furrow simply moved backwards.

The main conclusion to be drawn from these experiments is a confirmation of earlier work; that eggs continue to cleave when compressed unless the degree of flattening is considerable. Presumably the difference between the results of the flattenings in the two directions is due to the fact mentioned above; that the average degree of compression is less when the eggs are flattened at right angles to the polar axis. There are two further points to be drawn from these results. First, cleavage is delayed by compression before it is finally stopped. Secondly, eggs in Ca-free sea water are more susceptible to compression than eggs in ordinary sea water. Presumably this is due to the hyaline layer in ordinary sea water strengthening the cell membrane by acting as an elastic cover, and so lessening the degree of stretch of the membrane.

These results are probably compatible with all the main theories of cleavage so it is only worth pointing out here that they are quite consistent with the expanding membrane theory. Since the membrane is elastic, flattening the egg will increase the membrane tension, and it will therefore take longer for the tension to fall to zero as the membrane expands. This would account for the delay induced by compression. If the flattening is considerable the capacity for expansion of the membrane will not be sufficient to counteract the stretch of the membrane; the membrane will remain with a positive tension, and cleavage will not be possible. The reversions of the furrow might be due to a number of causes, but the most likely one seems to be stickiness between the microloops and the cell surface. This would allow the tension of the membrane within the loops to be higher than that of the furrow region. The furrow could therefore form, but when the tension in the membrane round it had fallen to a low value, the membrane would suddenly start to slide under the loop towards the poles, the tension would equalize all over, and the furrow would be pulled out.

It might be argued that the fact that eggs are able to cleave when compressed under a cover-glass (e.g. Danielli, 1952) is an argument against the expanding membrane theory. They are under a constant compressing force (unlike the eggs in the experiments above, which are under a constant deformation) and it might be expected that the membrane tension could never fall to zero. There are, however, two objections to this argument. First, the eggs may be supported by the remains of their asters. Secondly, a cover-slip pressing on an egg will only maintain a uniform tension over the membrane if the membrane can slip freely when it is against the glass. If the egg is flattened to any extent this probably does not occur.

PROPERTIES OF THE CELL MEMBRANE

It is well known that the cell membrane of sea-urchin eggs is highly elastic and has a considerable capacity to seal itself after damage even in Ca-free sea water (e.g. Chambers, 1924), but it is worth publishing a pair of photographs which show these properties in a striking fashion. Pl. 15, fig. 19, shows an egg which had just cleaved in Ca-free sea water and in which one blastomere had been cytolysed by a slicing cut. The other blastomere had been rested against the microloop and the needle had been carefully pushed through it. The needle had not punctured the membrane but had pushed it ahead so that the membrane finally joined up with itself on the far side of the egg. The needle was then pushed right through, so that the final result was a ring-shaped cell with a hole through the middle in which the needle lay. It was like a doughnut on a stick, and the egg could be rotated freely on the needle.

Pl. 15, fig. 20, shows that the cell membrane can be stretched considerably before it breaks. This was a cleaving egg in Ca-free sea water. When it was at the dumb-bell stage, it was placed against the microloop and pierced by the needle. The photograph shows the needle pushing out the cell membrane of one blastomere from the inside. The membrane had just given way at the tip of the needle when the photograph was taken, but before that it had remained stretched out for more than a minute. This shows that the material of the membrane must have an elastic limit (% stretch at the breaking point) of several hundred per cent.

SUMMARY

1. Chambers (1938) described an experiment in which he cut open one blastomere of a cleaving sea-urchin egg at the dumb-bell stage in isotonic KCl. The other blastomere contracted like a 'deflating balloon', and this has been taken by other workers as evidence of a positive membrane tension in the cleaving egg. This experiment has been repeated with other sea urchins in various media. It is concluded that this effect only takes place in one species of sea urchin, in an abnormal medium, and after it has suffered irreparable damage. It is not, therefore, legitimate to suppose that there is normally a positive membrane tension in a cleaving egg. It is found that eggs will continue to cleave with one blastomere in an irregular shape which indicates that, on the contrary, there is no membrane tension and no internal pressure. These are the conditions demanded by the 'expanding membrane' theory of cleavage.

2. It is found that the furrow of a cleaving egg will pass through a needle placed in its path. This result argues against a simple contracting ring in the furrow region being responsible for cleavage.

3. Chambers (1938) found that an egg will continue to cleave when its asters have been destroyed by stirring. This result has been confirmed by a similar experiment on a different species of sea urchin. This is crucial evidence against an astral mechanism of cleavage.

4. The effects of compressing cleaving eggs have been studied. It is found that compressed eggs continue to cleave unless the degree of flattening is considerable; that cleavage is delayed before it is finally stopped; and that eggs in Ca-free sea water are more susceptible to compression than eggs in ordinary sea water. These results are consistent with the 'expanding membrane' theory.

It is a pleasure to record my thanks to the Director and Staff of the Marine Station, Millport, and of the Stazione Zoologica, Naples, for their help in this work.

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EXPLANATION OF PLATES 14 AND 15

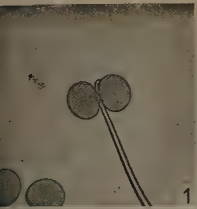
All eggs are of *Psammechinus miliaris* except in Pl. 14, figs. 6–12, which are of *Arbacia lixula*. OSW = ordinary sea water; CFSW = Ca-free sea water.

PLATE 14

Figs. 1, 2. Tearing one blastomere. CFSW. Other blastomere seals off. Interval of 3 min.
Figs. 3–5. Tearing one blastomere. CFSW. Other blastomere cytolyses. Intervals of 1 min.
Figs. 6–9. Tearing one blastomere. *Arbacia*. CFSW. No cytolysis. Intervals of 2 min.
Fig. 10. Tearing one blastomere. *Arbacia*. CFSW. Cleavage with only a small fragment left.
Fig. 11. Tearing one blastomere. *Arbacia*. Isotonic KCl. Both blastomeres cytolyse.
Fig. 12. *Arbacia* eggs placed in isotonic KCl at cleavage.
Figs. 13, 14. Passage of furrow through paraxial needle. OSW. Interval of 2 min.
Figs. 15–17. Passage of furrow through needle parallel to equator. OSW. Intervals of 2 min.

PLATE 15

Figs. 1–5. Destruction of asters by stirring. OSW. Intervals of 2 min. from figs. 2 to 5.
Fig. 6. Compression. Right angles to axis. OSW.
Figs. 7, 8. Compression. Right angles to axis. CFSW. Interval of 5 min.
Figs. 9–13. Compression. Parallel to axis. OSW. Intervals: figs. 10, 11, 1½ min.; figs. 11, 12, 2½ min.; figs. 12, 13, 7 min.
Figs. 14–18. Compression. Parallel to axis. CFSW. Intervals: figs. 15, 16, 1½ min.; figs. 16, 17, 1½ min.; figs. 17, 18, 6 min.
Figs. 19, 20. To show self-sealing and elasticity. CFSW.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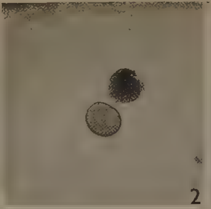
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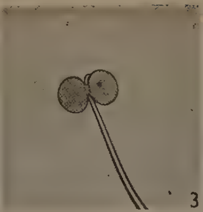
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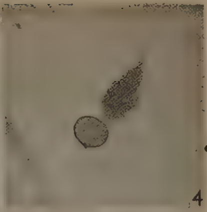
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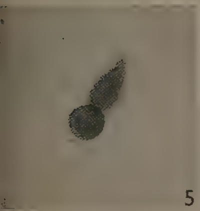
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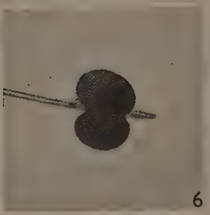
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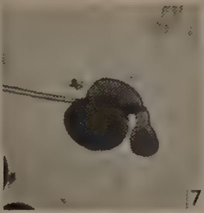
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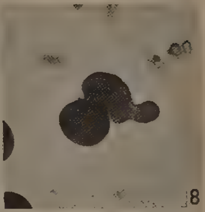
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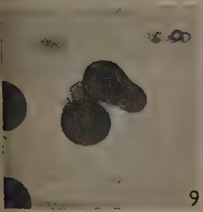
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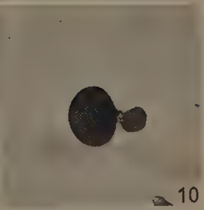
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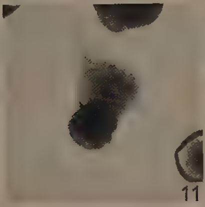
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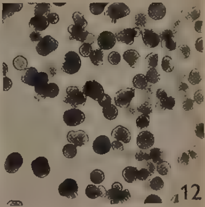
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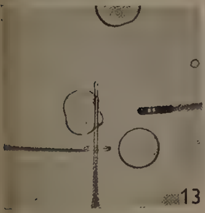
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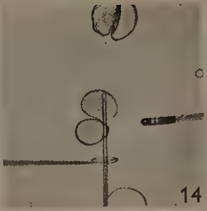
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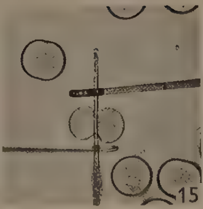
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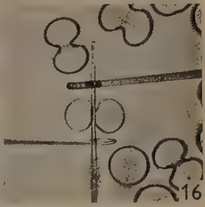
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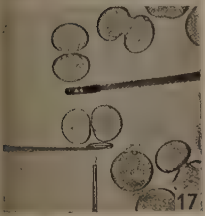
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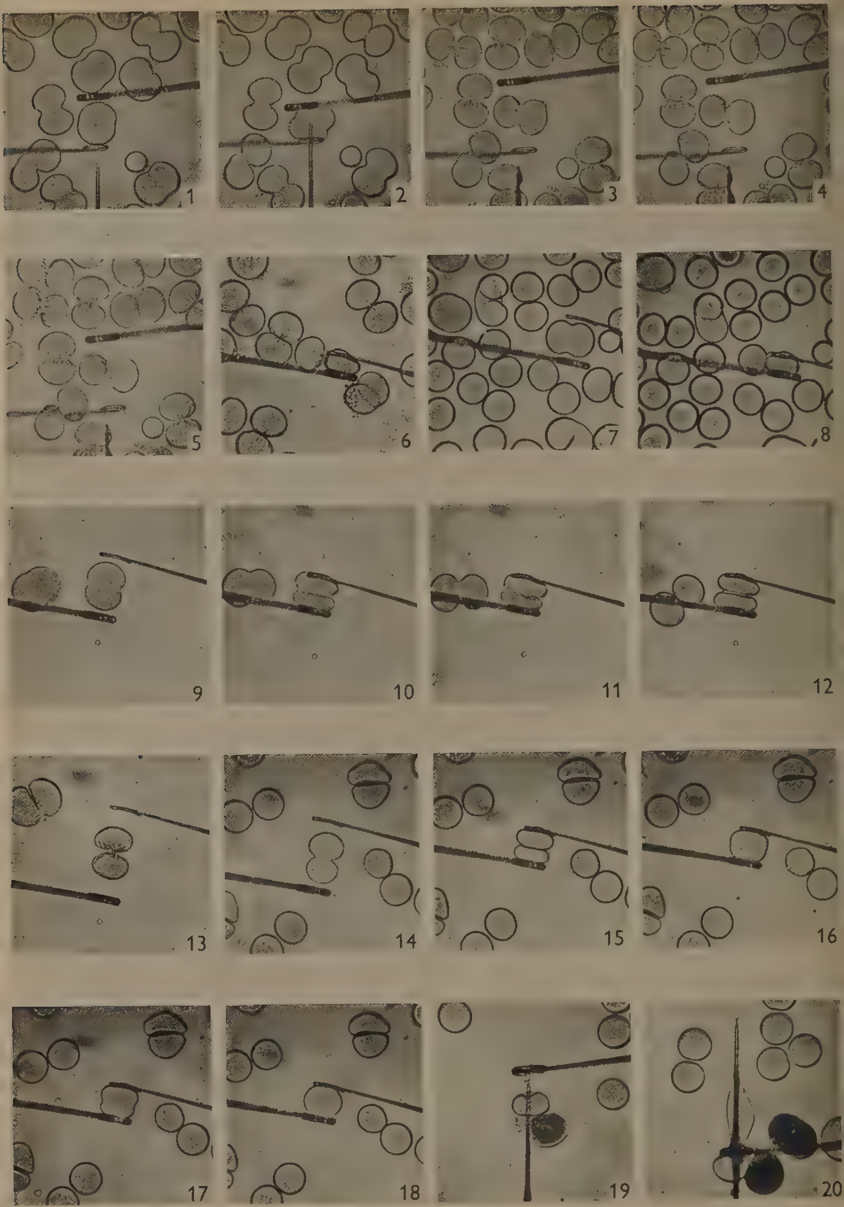
200μ Scale for all Pls. 14 and 15, except Pl. 14, figs. 6–12

100μ Scale for Pl. 14, figs. 6–11

400μ Scale for Pl. 14, fig. 12

ITCHISON—MICRODISSECTION EXPERIMENTS ON SEA-URCHIN EGGS AT CLEAVAGE



MITCHISON—MICRODISSECTION EXPERIMENTS ON SEA-URCHIN EGGS AT CLEAVA

THE DIURNAL RHYTHM OF ACTIVITY OF MAYFLY NYMPHS

By JANET E. HARKER*

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(*Received* 15 *October* 1952)

Moon (1940) has shown that nymphs of *Ecdyonurus* and *Baetis* move more during the night than the day under natural conditions, and he conjectured some indirect relationship between the stage of development and activity. This investigation has been undertaken as an attempt to discover the range of the diurnal periodicity of movement and the conditioning factors.

METHOD

Diurnal rhythm has been investigated in the movements of three species of mayfly nymphs: *Ecdyonurus torrentis* Kimmins, *Heptagenia lateralis* (Curtis), and *Baetis rhodani* (Pictet).

The simplest method of investigating activity has been used; in all but one experiment the animals were observed for periods of 10 min. at hourly intervals over the 24 hr.; each 10 min. period was divided into 300 sub-periods of 2 sec., and the number of animals seen to move in each sub-period was recorded. For reference purposes the movement of one animal in a 2 sec. period is termed an activity unit. In order to eliminate the systematic effect of any errors in observation due to fatigue of the observer (e.g. in the late evening) each 24 hr. series of observations was begun at a different time of day; and from one series to another the 10 min. period was chosen so as to cover a different part of the hour (e.g. from 10 to 20 min. past the hour, from half past to 20 min. to the hour, etc.), so that as far as possible there should be no period of the 24 hr. at which the animals had not been observed. Each experiment, involving a 24 hr. period of observation, was repeated five times under each set of conditions, unless otherwise stated, and each graph represents the activity during each hour calculated as the average of these five series.

Five animals were placed in a $6\frac{1}{2} \times 4\frac{1}{2}$ in. dish containing stones and water from the stream. The nymphs in two dishes only were observed in each 24 hr. period to allow some relaxation for the observer. Two sources of light were used, daylight and light from a 70 c.p. lamp giving an intensity of 200 f.c. measured by an 'Avo' light meter.

In order that the activity could be measured during the night the nymphs were painted on the thorax with a spot of white paint which was coated with a spot of

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luminous paint. The effect of the luminous paint on the nymphs was tested by comparing activity counts of control groups in very dim light, counts of the same insects before and after painting, and, to check any effect on other insects, counts of nymphs placed in dishes with small luminous stones. These experiments showed that the luminous paint did not appear to affect the activity of the nymphs.

During experiments carried out in the dark the stopwatch and record sheets were kept in a light-tight box containing a dim light, with a sunken slit in the top through which the stopwatch could be seen. The end of the box had been removed and was covered with a black cloth which allowed the entry of the hand, without the light being shown outside. Temperatures were taken in a control dish separated from the observation dish by a screen; readings of temperature during the day showed that there was no difference between the two dishes. The whole apparatus was placed on a table in front of an open window in a room in which there was no artificial lighting or heating except for the source of light aforementioned. In the later experiments a metronome was used instead of a stopwatch so that the attention of the observer was in no way distracted from the nymphs.

Except where otherwise stated, the nymphs were brought direct from the stream, and the experiments carried out immediately on arrival at the laboratory.

(*a*) *Natural daylight and darkness*

The first counts of activity of *Ecdyonurus torrentis* were carried out at half hourly intervals over 24 hr. in order that detailed observations could be made. Thereafter counts were only taken every hour over the 24 hr. periods, and comparison of the results showed this small number of readings to be adequate.

Five series of 24 hr. readings were taken in February, May and November; the animals being, respectively, May–March Stage XV, March–June Stage XV, and June–May Stage X nymphs (Harker, 1952). Fig. 1 shows the results of the activity counts of these five series.

Peaks of activity occurred at definite intervals, appearing consistently at 03.00–04.00, 05.00–07.00, 11.00–12.00, 16.00–17.00, 20.00–21.00, and 23.00–01.00 hr. The average length of each period of activity was 2 hr. Activity reached its peak rapidly, usually within an hour, and fell off slowly over a period of 2 or even 3 hr. The total activity recorded during the 10 min. periods over 24 hr. was 164 units in May, as compared with 112 and 116 units in February and November respectively.

These results indicate that the degree of activity varies according to the stage or brood of the nymphs. The variation in temperature in the 3 months suggests a connexion between temperature and activity. The fact that active periods were more frequent between 19.00 and 07.00 hr. than between 07.00 and 19.00 hr. suggests that variation in natural daylight is connected with the rhythm of activity. The times of the active periods were constant throughout the year, which further suggests that the connexion between light and the rhythm is not a direct one.

Further experiments were carried out in an attempt to correlate the rhythm with light variations, and with temperature variations which follow those of light.

(*b*) *Continuous light or darkness*

Observations were made in continuous light of animals taken directly from the stream, and of animals which had been kept in continuous light for 3 months. As animals kept in continuous darkness for long periods are difficult to feed, observations were made on nymphs only recently subjected to continuous darkness. The nymphs for these experiments were kept in a room in which the temperature was kept as close as possible to the outdoor temperature.

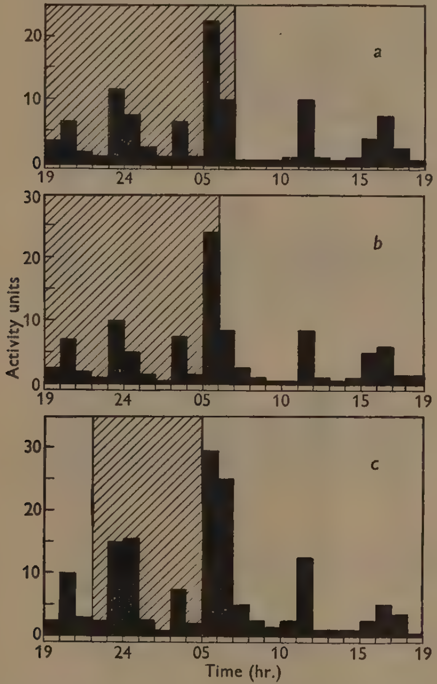

Fig. 1. The results of five series of activity counts of *Ecdyonurus torrentis* in: *a*, February; *b*, November; *c*, May. Hatched areas represent dark periods.

The results of five series of activity counts of nymphs under each of these conditions are given in Fig. 2*a*, *b*. It will be seen that in continuous light there was no change in the diurnal rhythm as seen in natural daylight and darkness. The total activity was increased from 129 to 189 units when the animals were first placed in continuous light, but this increased activity was reduced to a normal level of 119 units after 2 months. Continuous darkness did not alter the diurnal rhythm. The total activity was reduced to 109 units, the decreased activity taking place during the period of natural daylight.

(*c*) *Artificial reversal of light and darkness*

Animals taken from the stream were transferred to conditions of 'reversed' illumination (light from 19.00 to 07.00 hr., and darkness from 07.00 to 19.00 hr.). The dark period was probably darker than is normally experienced.

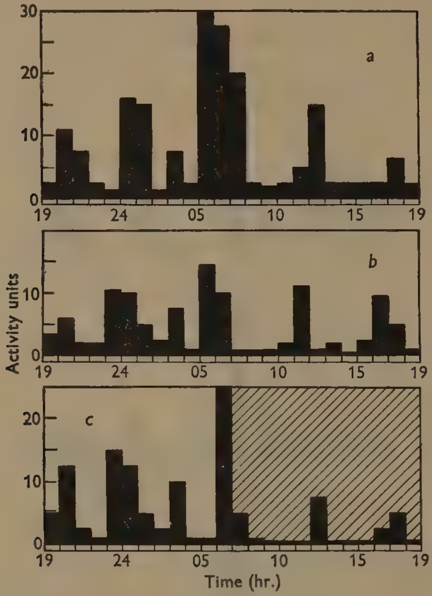

Fig. 2. The results of five series of activity counts of nymphs of *Ecdyonurus torrentis*: *a*, in continuous light; *b*, after being exposed to continuous light for 1 month; *c*, in 'reversed' light and darkness.

Results are shown in Fig. 2*c*. Activity increased during the light period to 93 units compared with 65 units under natural conditions. There was also a fall in activity in the dark period to 33 units compared with a 'natural' 51 units. After 2 months the activity had returned to normal.

The rhythm of activity was in no way altered by conditions of 'reversed' illumination.

(*d*) *Effect of constant temperature*

Fig. 3 shows the activity of nymphs which had been kept at a constant temperature of 20 ± 1° C. for 3 weeks. The actual observations were not made in the constant temperature room (C.T.R.), but under the conditions described in section (*a*). To check the effect of removing the nymphs from the constant temperature room observations were made over limited periods during 5 days in the C.T.R.

The nymphs removed from the C.T.R. showed no change in their rhythm of activity, but their total activity was higher than under normal conditions, being 138 units. The nymphs in the C.T.R. (the rhythm of activity of these nymphs is shown in Fig. 3*b*) likewise showed no change in their rhythm of activity, but their total activity was even higher than that of the previous set.

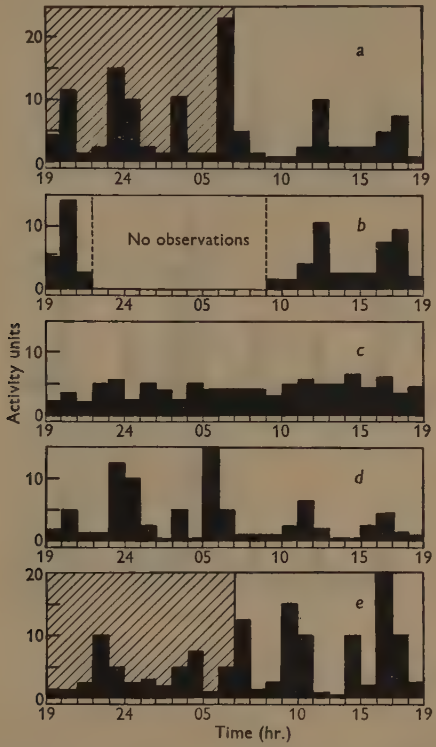

Fig. 3. The results of five series of activity counts of *Ecdyonurus torrentis*: *a*, after being kept at 20° C. for 3 weeks (readings not taken at 20° C.); *b*, after being kept at 20° C. for 1 week (readings taken at 20° C.); *c*, after being kept in continuous light from oviposition; *d*, the same nymphs after exposure to natural daylight and darkness for 1 day and then being left in continuous light for 1 week. *e*, after being kept in continuous light from oviposition, being exposed to darkness from 10 a.m. to 4.0 p.m. G.M.T. for 1 day, and then left in natural daylight and darkness.

(*e*) *Effect of continuous light from oviposition*

A number of eggs were taken immediately after oviposition and kept in continuous light until 7 months after hatching. Five groups of fifteen nymphs were observed at intervals over a period of some weeks, and, as can be seen in Fig. 3*c*, it was found that the rhythm had either been broken or had failed to develop. There was no rhythm in the activity at all, and the total activity had dropped considerably.

The exposure of these nymphs to natural daylight and darkness for 1 day was sufficient to impress a rhythm upon them. Fig. 3*d* shows the activity a week after the nymphs had been transferred to continuous light again. The total activity is still low.

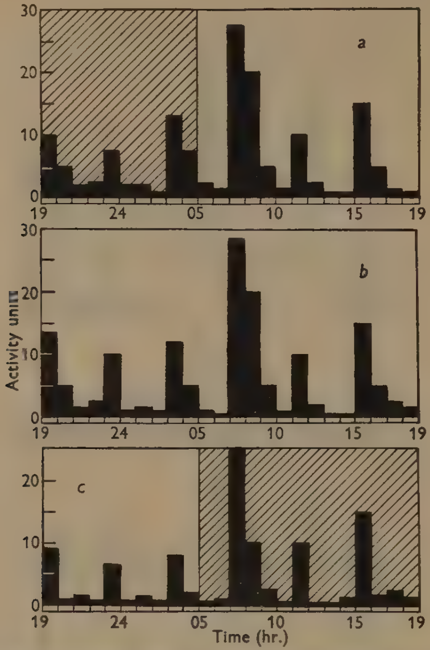

Fig. 4. The results of five series of activity counts of nymphs of *Heptagenia lateralis*: *a*, in conditions of natural daylight and darkness; *b*, in continuous light; *c*, in 'reversed' light and darkness.

Another group of nymphs, bred in continuous light, was exposed to darkness from 10.00 to 16.00 hr. for 1 day, and then transferred to the same conditions of natural daylight and darkness as a control group of nymphs taken directly from the stream. Fig. 3*e* shows that the experimental group took up a rhythm of activity, but not the same rhythm as the stream group. The peaks of activity for the former group are closer together between 10.00 and 16.00 hr., when they were first exposed to darkness, than in the normal group. This experiment further indicates that the original rhythm of light and darkness to which the eggs or nymphs are exposed impresses the rhythm of activity.

(*f*) *Rhythm in ecologically related species*

Observations of activity of *Heptagenia lateralis* and *Baetis rhodani* showed that both of these species had a type of rhythm similar to that of *Ecdyonurus torrentis*.

Fig. 4 gives the results of five experiments on *Heptagenia lateralis* in natural daylight and darkness. The main peaks of activity occur at 03.00, 07.00, 10.00, 14.00, 16.00, 18.00 and 23.00 hr.; again the intervals between the active periods are less in the early morning than for the rest of the 24 hr. The highest activity is regularly shown at 07.00 hr., and the second highest at 15.00 hr.

It is interesting to note that the rhythm of activity is in the same form as that of *Ecdyonurus torrentis*. Specimens were collected from another stream and observed through a series of 24 hr. periods. Fig. 6*b* shows that these had the same rhythm of activity as the previous specimens, although the peaks of activity occurred at slightly different times of day.

Baetis rhodani (Fig. 5) showed a rhythm similar in form, but not as marked, as the other two species. The peaks of activity in this species occurred at the same time as peaks in *Ecdyonurus torrentis*.

Experiments were carried out under 'reversed' conditions of illumination with both *Heptagenia lateralis* and *Baetis rhodani*, and again the same effects were shown as in *Ecdyonurus torrentis*. The total activity was higher during the light period, but the rhythm remained unaltered (Figs. 4*c* and 5*c*). Nymphs in continuous light kept their rhythm (Figs. 4*b* and 5*b*), but the general level of activity was increased.

DISCUSSION

The greater part of the work published on diurnal rhythms of other insects has indicated that the daily rhythm in light intensity is the most important factor conditioning the distribution of activity. Temperature, too, has been shown to affect the rhythm; and it has been suggested that in fact activity cycles are controlled not by one, but by a number of factors acting simultaneously.

These experiments carried out on mayfly nymphs suggest that neither light nor temperature has a direct effect on the diurnal rhythm once that rhythm has been established. The evidence from the nymphs which had been subjected to continuous light from the egg stage suggests that the rhythm is imposed very early in life. There is, however, no indication of the way in which the diurnal rhythm of activity is related to the natural rhythm of daylight and darkness. On the one hand the three broods of *E. torrentis* from the same stream showed the same rhythm, although, being hatched at different times of the year, they could have been exposed to different conditions of natural light and darkness. On the other hand, the rhythms of the nymphs from two different streams did not coincide, although it might be supposed that the difference in the light cycle between the two streams would not be greater than the difference within the one stream over the period from March to July when the three broods emerge.

It is suggested that the reaction to the light-darkness rhythm is in some measure inherent. As the chances of the mayfly fauna of one stream mating with another are limited by the short adult life and the occurrence of swarming, there may be a tendency for each species in a stream to develop its own rhythm within its specific range.

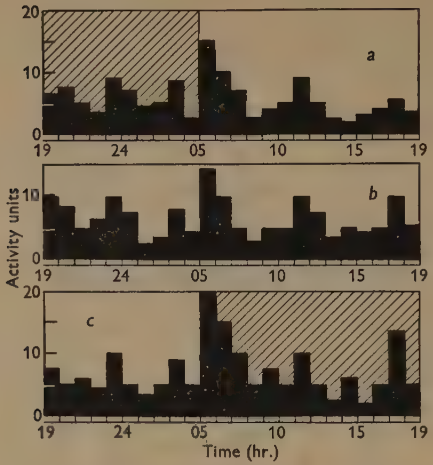

Fig. 5. The results of five series of activity counts of nymphs of *Baetis rhodani*: *a*, in conditions of natural daylight and darkness; *b*, in continuous light; *c*, in 'reversed' light and darkness.

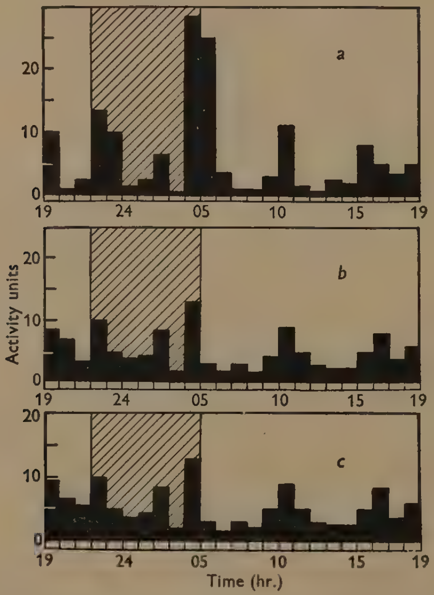

Fig. 6. The results of five series of activity counts of nymphs of: *a*, *Ecdyonurus torrentis*; *b*, *Heptagenia lateralis*; *c*, *Baetis rhodani*. All taken from another stream in May.

SUMMARY

1. The activity of *Ecdyonurus torrentis* Kimm., *Heptagenia lateralis* (Curt.) and *Baetis rhodani* (Pict.) has been measured over a series of 24 hr. periods by recording their movements over 10 min. periods every hour.

2. Diurnal rhythms have been found to occur under conditions of natural daylight and darkness in all three species, but the periods of activity occur at different times, according to the species.

3. These rhythms are maintained under conditions of continuous light or darkness and under conditions of 'reversed' illumination.

4. In *Ecdyonurus torrentis* the rhythm is not seen in nymphs bred from eggs in continuous light, but appears abruptly, in almost fully developed form, after exposure of the nymphs to natural daylight and darkness for a period of no more than 24 hr.

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THE INORGANIC CONSTITUENTS OF THE SEA-URCHIN EGG

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(*Received* 27 *February* 1953)

INTRODUCTION

Previous work. The most detailed investigations into the inorganic constituents of the sea-urchin egg are those of Page (1927) and Białaszewicz (1929). Their results, together with those of certain other workers in this field, are given in Table 1 and, in accordance with modern practice, the concentrations of the various substances have been converted into millimoles (mM) per kilogram of water in the eggs. The dry weight of sea-urchin eggs is about 24% of the wet weight (Wetzel, 1907; Ephrussi, 1933; Ballentine, 1940; Hutchens, Keltch, Krahl & Clowes, 1942).

Table 1. *Inorganic constituents of sea-urchin eggs, in mM/kg. water in eggs*

	Sea water, chlorinity 19·00‰	*Arbacia punctulata* (Page, 1927)	*A. punctulata* (Harvey, 1932)	*A. lixula* (Białaszewicz, 1929)	*A. lixula* (Monroy-Oddo, 1946)	*Paracentrotus lividus* (Białaszewicz, 1929)	*Strongylocentrotus droebachiensis* (Malm & Wachtmeister, 1950)	*Psammechinus miliaris* (Malm & Wachtmeister, 1950)
Sodium	475	321	—	280	—	25	316	343
Potassium	10	354	96	162	—	245	200	266
Calcium (+Sr)	10	269	38	16	17	14	—	—
Magnesium	54	1044	17	41	21	28	—	—
Chloride	554	30	—	384	—	371	298	332
Sulphate	29	(0·0301)	—	—	—	—	—	—
Total phosphorus	—	181	—	132	—	142	110	148

The molalities of the commoner ions in sea water are also given in Table 1. The great discrepancies between the values obtained by different workers for the inorganic constituents of sea-urchin eggs are a sufficient reason for re-examining this subject. It seems, for example, unlikely that the magnesium content of two different batches of unfertilized eggs of *Arbacia* should vary by some 6100%; that the chloride content of different species should differ by about 1300%; or even, perhaps, that the sodium content of different species should differ by 1400%.

Apart from the results given in Table 1, a certain number of observations have been made on the concentration and exchange rates of particular substances in sea-urchin eggs. Runnström, for example, said in 1925 that potassium was at least eight times as concentrated in the eggs of *A. lixula* as in sea water. Many of these observations have, however, been published as brief notes and, in the absence of subsequent papers of normal length, it is difficult to know how much weight should be attached to these preliminary communications. This applies to three notes on the penetration of ^{32}P (Abelson, 1948; Brooks & Chambers, 1948; Chambers, E. L., Whiteley, Chambers, R. & Brooks, 1948) and to two notes on the uptake of ^{42}K (Chambers, 1949; Chambers, White, Jeung & Brooks, 1948). Shapiro & Davson (1941) state, also in a brief note, that the eggs of *A. punctulata* contain about twenty times as much potassium as there is in sea water, but the methods by which they arrived at this conclusion are not given.

Interstitial water. All the results quoted in Table 1 were obtained by centrifuging the eggs and analysing them after removal of the supernatant suspending medium. There was, therefore, an unknown amount of interstitial sea water or suspending medium between the eggs, and it is incorrect to assume that this interstitial liquid can be ignored. In some preliminary experiments using the Trypan Blue method (see later), which are not of sufficient importance to include in the Results Section, it was found that the amount of interstitial sea water remaining after centrifugation of unfertilized eggs of *Echinus esculentus* for 15 min. at × 12,000 g. varied from 2% to 9%. This source of error may not be of great importance when the substance under examination has about the same concentration in the eggs as in sea water. But if it is much more concentrated in the latter than the former, as in the case of sodium according to Białaszewicz, or chloride according to Page, a knowledge of the amount of fluid between the eggs is essential if the analyses are to have any meaning. If, as Page claimed, the concentration of chloride in eggs is one-eighteenth of what it is in sea water, and if, after centrifugation, there remains 5% of interstitial sea water which is ignored, the apparent concentration of chloride in the eggs will be 185% too high. Some investigators tried to get round this difficulty by washing and suspending the eggs in isotonic glucose solution (Blanchard, quoted by Harvey, 1932), isotonic lithium nitrate (Białaszewicz, 1929), or a molar solution of urea (Monroy-Oddo, 1946). In the absence of experimental evidence that these treatments do not cause an outward diffusion of electrolytes from the eggs, these methods of attempting to circumvent the problem of interstitial sea water are unsatisfactory. Malm & Wachtmeister (1950) may have realized the importance of this problem; they state (p. 448) that 'almost all sodium chloride found in the unfertilized eggs originates from the intercellular water and not from the eggs themselves'. In justification of this contention they say that Białaszewicz showed in 1927 that 'unfertilized sea-urchin eggs contain but little sodium'; but reference to Table 1 shows that in 1929 Białaszewicz came to an entirely different conclusion, so far as the eggs of *Arbacia lixula* were concerned.

One can, of course, use the analyses based on centrifuged eggs to establish maximum or minimum concentrations, according to whether the substance under examination is less or more concentrated in the eggs than in the sea water. This method of establishing concentration limits, which involves the assumption that high-speed centrifugation does not affect the concentration of substances in the egg, has been used in pilot experiments done before those described in this paper. There are two other methods of estimating the amounts of sea water and eggs in a suspension. The first is to add a known amount of some non-penetrating solute to a known volume of egg suspension, mix, and measure the solute's concentration in the suspending medium. The difference between the concentration of the solute, assuming no eggs in the suspension, and the observed concentration enables the volumes of eggs and sea water in the suspension to be calculated. This method was tried out with two different non-penetrating solutes. The first was a levan polysaccharide of high molecular weight obtained from Italian rye-grass, kindly supplied by Dr J. Beattie. This method was unsatisfactory because of the high reducing activity of the eggs. A small amount of egg cytolysis, which is inevitable in experiments of this type, interfered with the colorimetric determinations to a degree which made reasonable precision unattainable. The second solute was Trypan Blue. Although this substance does not penetrate the eggs, it appeared in some cases to become adsorbed on to the egg surfaces and on debris. In addition, the Trypan Blue method did not give reproducible results, and it was therefore decided to abandon these methods and estimate the number of eggs in the suspension, together with their volumes. The procedure is described in the next section.

Precision of measurements. Little attention has been paid to this question, though it is difficult to know what importance should be attached to measurements unless some indication is given of the errors associated with them. In two cases (Blanchard, quoted by Harvey, 1932, and Monroy-Oddo, 1946) estimates are followed by plus or minus some figure, which may mean the standard error of the mean of some number of estimates. But the practical object of working out standard errors is to define an interval which encloses the true value with some assigned probability. When estimates are based on very few measurements, the standard error cannot be used for an accurate determination of the interval which encloses the true value unless the distribution under consideration is normal, or if some transformation is known to make it normal. For this reason and because of the interstitial-water problem mentioned above, it has not been thought necessary or desirable to examine previous results in great detail, nor to try to explain the discrepancies between the results of different workers.

EXPERIMENTAL PROCEDURE AND CALCULATIONS

Estimation of volume of egg suspension and total egg volume. There are three parts to these estimations: first, determination of egg radii, from which the mean volume $\bar{v}$ of an egg and its variance $V(\bar{v})$ are obtained; secondly, estimation of the number of eggs in the suspension under examination, together with the precision of the estimate; thirdly, estimation of the volume of the egg suspension under examination. The eggs of *Paracentrotus lividus* do not flatten under gravity, so that $\bar{v}$ and $V(\bar{v})$ were determined by measuring two diameters, at right angles to each other, of fifteen to twenty-five eggs. The number of eggs in the suspension was estimated as follows: the parent egg suspension, which consisted of jelly-free unfertilized eggs (the jelly being removed by the usual treatment with acidified sea water) and sea water in a boiling tube (diameter 2·3 cm., length 15 cm.), was gently inverted ten times to suspend the eggs uniformly in the sea water. A known volume of this parent suspension was then removed with a pipette and diluted to 50 ml. in a stoppered flask with sea water containing 5% formalin. After agitation to suspend the eggs uniformly, samples of known volume were removed from the flask and the number n of eggs in these samples counted. A similar method was described by Shapiro in 1935. The volume of the counting tube was 0·096 ml. in all experiments. In a typical experiment eight egg counts were made, $\bar{n}$ being 163† and $V(\bar{n})$ 16·786. In this experiment $V(n)$ equalled 134·288, a value which is not inconsistent with a Poisson distribution of n, though this was not always the case. Estimation of the volume of the egg suspension upon which the chemical analyses were done was based on a series of weighings, from which, by calculation of the appropriate densities, the volume of the egg suspension was obtained. The original egg suspension was concentrated by very gentle centrifugation, for about ½ min. at less than × 50 g., after which most of the supernatant sea water was removed by suction. After determination of the volume of this concentrated egg suspension, it was evaporated in a steam oven and the dried material (eggs + sea water) analysed (see pp. 538–40).

Estimation of concentration of substances in eggs. Let V_F = final volume of the suspension, K_F = amount of potassium (for example) in V_F, K_{SW} = concentration of potassium in sea water, and k = the unknown concentration of potassium in the eggs. Let $x = \bar{n}\bar{v}$ and A be the dilution factor used when estimating $\bar{n}$. Then

$$K_F = K_{SW}(V_F - Ax) + kAx. \qquad (1)$$

Solving for k,

$$k = \frac{K_F - K_{SW}V_F}{Ax} + K_{SW}. \qquad (1\cdot1)$$

Bearing in mind that if $u = f(z)$, $V(u) = \{f'(z)\}^2 V(z)$,

$$V(k) = \frac{(K_F - K_{SW}V_F)^2}{(Ax)^4} A^2 V(x), \qquad (2)$$

where $V(x) = \bar{n}^2 V(\bar{v}) + \bar{v}^2 V(\bar{n})$.

Equation (2) gives the precision of k. In practice, however, a number of estimates of the concentration of potassium (and other substances) were made; so that in the case of potassium, for example, we have a series of estimates k_i, each with variance $V(k_i)$, $i = 1, \ldots, N$. As the errors in chemical analysis were small compared with the errors involved in estimating $\bar{n}$ and $\bar{v}$, and as inter-batch variations were large compared with intra-batch errors (see later), the best estimate of k, k^*, is simply $(1/N)(\Sigma k_i)$, with variance $V(k^*) = \Sigma(k_i - k^*)^2/N(N-1)$. Inter-batch variation may be quantitatively estimated by subtracting $\overline{V(k_i)}$, the average value of the individual batch variances, from $V(k) = \Sigma(k_i - k^*)^2/(N-1)$. k^* and $V(k^*)$ may be used to define an interval within which the true mean of the observed values of k

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† $\bar{n}$ must be multiplied by the appropriate dilution factor to obtain the number of eggs in the parent suspension.

lies with any desired probability, while the estimate of inter-batch variation may be used to define another interval within which a particular k_i will lie, with any desired probability.

Chemical analyses

The methods used for the estimation of cations were similar to those described by Robertson & Webb (1939), who developed reliable micro-methods for the analysis of 1 ml. of sea water or body fluid, with a maximum error of 2%. Minor modifications and details of the other methods used are described below.

Ashing. The egg suspension, whose volume was 2–7 ml., was ashed after evaporation to dryness with 2 ml. concentrated H_2SO_4, re-ashed if necessary, and taken up in a small quantity of distilled water with the addition of 0·3 ml. concentrated HNO_3. After making up to 10 ml., portions were taken for determination of the various cations.

Sodium (*zinc uranyl acetate method*). Phosphate interferes with the sodium analysis since it precipitates as zinc uranyl phosphate. A small quantity of finely powdered $Ca(OH)_2$ was therefore added to the solution for analysis and, after shaking several times while standing for 30 min., the precipitate of calcium phosphate and excess $Ca(OH)_2$ was removed by centrifugation. A portion of the supernatant was then taken for analysis.

Potassium (*silver cobaltinitrite method*). The precipitate was not left to stand overnight but was kept in the refrigerator for 4 hr. Ice-cold water was used for washing the precipitate instead of a saturated solution of the potassium salt.

Magnesium (*8-hydroxyquinoline complex method*). As an adaption to smaller quantities (0·2–0·3 mg.) the whole magnesium analysis was done in a centrifuge tube. 1 ml. of oxine was added to the solution (about 8 ml.), followed by one drop of NH_4OH (50%, v/v), and the complex, after precipitation at 70–80° C. with stirring, allowed to stand for 10 min. at about 90° C. After centrifugation and removal of the supernatant, the precipitate was washed with 2% NH_4OH, re-dissolved in three drops of 2N-HCl and re-precipitated by the addition of 0·3 ml. reagent followed by two drops of 50% NH_4OH, the conditions being maintained as in the first precipitation. The titration was carried out in the usual way.

Calcium. This was estimated as calcium oxalate, the latter being titrated with standard ceric sulphate. The first precipitate of calcium oxalate was re-dissolved and then re-precipitated to remove magnesium in the original precipitate.

Chloride. A more direct method was used than that of Robertson & Webb. After cytolysing the eggs by vigorous shaking with distilled water the solution was deproteinized with Somogyi's zinc sulphate-barium hydroxide reagent (1945). The precipitate was separated by centrifugation and filtration, the solution made up to 50 ml. and portions titrated by Volhard's method. After adding HNO_3 and excess standard $AgNO_3$ solution (about 0·02N) to the aliquot and centrifuging off the precipitated halides, it was back-titrated with NH_4CNS (about 0·01N, previously standardized against the $AgNO_3$ solution), using 1 ml. of saturated ferric alum solution as indicator. The total volume was less than 20 ml.

Sulphate. Robertson & Webb's method was not used. After cytolysis with distilled water followed by de-proteinization with 5% $HgCl_2$, the sulphate was determined gravimetrically as $BaSO_4$, taking the standard precautions for this method and weighing the precipitate on a micro-balance.

Phosphorus. For determination of phosphate fractions the egg suspension was cytolysed with distilled water and made up to a known volume. The total phosphorus was determined on an aliquot which was transferred to a boiling tube, evaporated and ashed with H_2SO_4. After clearing with 100 vol. M.A.R. H_2O_2 and neutralization with NaOH, the sample was made up to a known volume, and the phosphate was determined by the Fiske-Subbarow method (1925) using the Spekker absorptiometer and red filters (Ilford 608). A second aliquot was treated with ice-cold trichloroacetic acid (final concentration 10%) and, after centrifugation, the inorganic phosphate was determined directly on one aliquot of the supernatant. The acid-soluble phosphorus was determined on a second aliquot after ashing, clearing and neutralization.

Accuracy of the analytical results. Robertson & Webb claimed that the errors associated with their methods did not exceed 2%, and inspection of their tables shows that the errors were often much less than this. During this investigation 1 ml. samples of sea water, whose chlorinity had been determined by the standard Knudsen technique, were taken through the ashing procedure and analysed. The accuracy of the results can be assessed by a comparison of the ionic ratios obtained with those given by Lyman & Fleming (1940). The results are given in Table 2.

Table 2. *Ratio of ions in sea water to chlorinity.*

Cl, Volhard (micro), 18·46‰. Standard Knudsen Method, 18·44‰.		
Ion	Found	Best value
Potassium	0·02043	0·0200
Sodium	0·5548	0·5556
Calcium (+Sr)	0·2113	0·2152
Magnesium	0·06701	0·06695
SO_4	0·1384	0·1394

Regardless of the absolute concentrations of the salts, the ratios between the common ions in sea water are virtually constant. This constancy of composition is not only the basis of the chlorinity-salinity density relations; it also provides a means of estimating the concentration of all the so-called major constituents when one is known. The major 'constituent' most easily determined with accuracy is the silver precipitating halides and their concentration is expressed as chlorinity. Chlorinity is defined by Knudsen as the weight in grams (*in vacuo*) of the chlorides contained in 1 kg. of sea water (*in vacuo*) when all the bromides and iodides have been replaced by chlorides, the values being calculated using Knudsen's Tables and Copenhagen Normal Water as standard. It is desirable to maintain an unvarying standard for chlorinity independent of atomic weights so that the apparent chlorinity of the oceans will not vary with changes in atomic weights. Jacobsen & Knudsen (1940) have established a permanent standard (in terms of weight of silver) identical with the previous one but independent of the store of Normal Water at Copenhagen. The ionic ratios are conveniently expressed in relation to the chlorinity. Strictly speaking, the ratios only hold good at chlorinities in the neighbourhood of 19‰, although the divergences are small for comparatively wide changes of salinity.

Webb (1938) has pointed out that strontium is chemically indistinguishable from calcium in sea-water analyses by the usual techniques, and that this leads to high values for the apparent calcium content. The ratio $(Ca+Sr)/Cl$ is 0·02152.

In the experiments described in this paper (apart from the check experiments already mentioned), the sea-water concentrations were calculated from Lyman & Fleming's ratios after accurate salinity determinations using a standard Knudsen burette and Knudsen's Tables. When concentrations were required in terms of grams per litre, the density was also calculated by the use of these tables. The chlorinity of Roscoff sea water was found not to vary significantly from 19·37‰ during these experiments.

To investigate the accuracy of the phosphate fraction estimations, duplicate analyses were done on different portions of an egg suspension derived from one sea-urchin; the results are given in Table 3.

Table 3. *Accuracy of phosphate estimations, mg./ml.* (Echinus esculentus). *Duplicate analyses,* i *and* ii, *were done on two samples of eggs from one sea-urchin*

	Total P		Acid-soluble P		Inorganic P	
Analysis ...	i	ii	i	ii	i	ii
Sample 1	2·97	3·02	1·64	1·64	0·91	0·93
Sample 2	2·94	2·98	1·72	1·72	1·05	1·04

RESULTS

Table 4 gives the results of the analyses, in mg./ml. eggs and in millimoles per kg. of water in eggs, of unfertilized, jelly-free eggs of *P. lividus*. The molalities of the same ions and radicals in Roscoff sea water are included for comparison. There are, perhaps, four features of these analyses which require special mention: first, sodium is nine times as concentrated in sea water as in the eggs; secondly, potassium is twenty-one times as concentrated in the eggs as in sea water; thirdly, there is nearly seven times as much chloride in sea water as in the eggs; and fourthly, there appears to be a large anion deficit in the eggs, which contain 292 m.equiv. of cations per kg. of water in the eggs and 194 m.equiv. of anions. In arriving at the anion figure, 1·5 charges have been allotted to each phosphorus atom, which implies that the phosphorus content of the egg consists of equal amounts of HPO_4 and H_2PO_4 at a pH of about 7 (see, for example, Needham & Needham, 1926). These assumptions certainly exaggerate the contribution of phosphate to electroneutrality because the acid-soluble phosphorus only accounts for somewhat more than half the total phosphorus present in the eggs. The proportions of acid-soluble and inorganic phosphorus are shown in Table 5. The pooled estimate of total phosphorus, together with the precision of this estimate, given in Table 4, is derived from the data in Table 5.

Table 4. *Inorganic constituents of unfertilized eggs of* Paracentrotus lividus

	c	S.E. of c	n	V	mM	Sea water (mM)
Sodium	0·9996	0·2939	10	0·8626	52	485
Potassium	6·7917	0·3970	9	1·1653	210	10
Calcium	0·1338	0·0192	7	0·0499	4	11
Magnesium	0·2124	0·0860	6	0·2066	11	55
Chloride	2·3354	0·9568	3	1·5261	80	566
Sulphate	0·4441	0·1541	4	0·2967	6	29
Total phosphorus	2·0974	0·0497	3	—	—	—

c, mg./ml. eggs. This is the best estimate, e.g. k^*, see p. 537.

S.E. of c, standard error of c, i.e. $\sqrt{[V(k^*)]}$.

n, number of independent determinations.

V, inter-batch variance. In the case of phosphorus the inter-batch and intra-batch variations were of the same order, so that c was calculated by the formulae

$$k^* = \Sigma\{I(k_i)\,k_i\}/\Sigma I(k_i) \quad \text{and} \quad I(k^*) = \Sigma I(k_i),$$
$$\text{where } I(p) = 1/V(p).$$

mM, c in terms of millimoles per kilogram of water in the eggs (dry weight of eggs, 24%; density, 1·09).

Sea water (mM), millimoles per kilogram of water, chlorinity 19·37‰.

Table 5. *Phosphorus fractions, in mg./ml., unfertilized eggs of* Paracentrotus lividus

Sea-urchin no.	Acid-soluble P	Inorganic P	Total P
i	1·19	0·69	2·12
ii	1·07	0·61	2·02
iii	1·19	0·79	2·19

DISCUSSION

The asymmetrical distribution of ions between the inside of a cell and the external medium has been the subject of a great number of inquiries and of an almost equal amount of discussion and speculation. The sea-urchin egg is similar in several respects to other tissues, such as nerve, muscle and erythrocytes, which have received much more attention and about which much more precise information is available. The mechanisms responsible for the distribution of ions between these cells and their environments have been discussed in great detail, for example, by Boyle & Conway (1941), Ussing (1949) and Solomon (1952). We therefore do not propose to recapitulate the various arguments but to examine certain fundamental questions so far as the incomplete information permits. The fundamental questions are:

(1) *How is electroneutrality maintained in the egg?* Amino-acids such as glutamine probably make a significant contribution to the anion deficit (Kavanau, 1953).

Bicarbonate is unlikely to contribute to the missing anion in view of the reported pH of the egg interior (Needham & Needham, 1926).

(2) *Are the observed concentrations maintained by active processes?* If the egg is in a steady state and no active work is being done to maintain the observed differences between the concentrations of potassium and chloride inside and outside the egg, but an active process is responsible for the asymmetrical distribution of sodium, the Donnan equilibrium requires that $[K]_i/[K]_o=[Cl]_o/[Cl]_i$, where the square brackets refer to concentrations and the subscripts i and o refer to the inside and outside of the egg. If an active process is responsible for the asymmetrical distribution of potassium and not for the sodium or chloride distribution, $[Na]_i/[Na]_o=[Cl]_o/[Cl]_i$, while if chloride alone is actively transferred, $[K]_i/[K]_o=[Na]_i/[Na]_o$.

Table 6

Active transport of	Donnan equilibrium	Ratio
Sodium	$\left.\begin{array}{l}[K]_i\,[Cl]_i = 16{,}800 \\ [K]_o\,[Cl]_o = 5{,}660\end{array}\right\}$	3·0:1
Potassium	$\left.\begin{array}{l}[Na]_i\,[Cl]_i = 4{,}160 \\ [Na]_o\,[Cl]_o = 274{,}510\end{array}\right\}$	1:66·0
Chloride	$\left.\begin{array}{l}[K]_i\,[Na]_o = 101{,}850 \\ [K]_o\,[Na]_i = 520\end{array}\right\}$	195·9:1

Table 6 gives the results of substituting the observed concentrations of sodium, potassium, and chloride in these relationships. It shows that if, on the basis of the existing evidence, a decision had to be taken in support of one ion being the subject of active transport, the decision would be in favour of the egg actively pumping out sodium. But the available evidence is far too scanty to warrant such a conclusion at present.

Lack of information about the permeability of the egg surface to potassium, sodium or chloride makes it impossible to say anything about the work which might be necessary to maintain the observed concentration differences. In addition, Chambers, White, Jeung & Brooks (1948) make the 'tentative assumption' (p. 252) that only 20% of the intracellular potassium is readily exchangeable with sea water in the case of the unfertilized eggs of *Arbacia punctulata* and *Strongylocentrotus purpuratus*. In the absence of more detailed information, particularly about the possibility of contamination of the ^{42}K used with ^{24}Na, and about the difficulties in interpreting experiments to demonstrate the existence of 'bound' ions (see Keynes, 1951), it may be premature to accept the findings in this preliminary note of Chambers and his co-workers.

SUMMARY

1. The principal inorganic constituents of the unfertilized egg of *Paracentrotus lividus* have been analysed by chemical methods. The results of the analyses, in millimoles per kg. of water in the eggs (dry weight of eggs, 24%; density, 1·09), were:

Sodium	52 (485)	Magnesium	11 (55)
Potassium	210 (10)	Chloride	80 (566)
Calcium	4 (11)	Sulphate	6 (29)

The figures in brackets are the concentrations of the same substances in Roscoff sea water, chlorinity 19·37‰, in the same units.

2. The total phosphorus content of the eggs was about 2 mg./ml. eggs, somewhat over half of this being acid-soluble phosphorus.

We are greatly indebted to the Director and staff of the Station Biologique, Roscoff, where these experiments were carried out, to Mr D. R. Cox of the Statistical Laboratory, Cambridge, for advice, and to Mr M. J. Hubbard for his technical assistance. One of us, R., is in receipt of a grant from the Medical Research Council for a laboratory assistant.

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THE MOULTING FLUID OF THE CECROPIA SILKWORM

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(*Received* 26 *February* 1953)

INTRODUCTION

For a period prior to each moult an insect is surrounded by two cuticles—the old cuticle about to be shed enveloping the new cuticle in process of formation. As first noted by Malpighi (1669), the space between the old and new cuticles is at this time occupied by a transparent fluid, the so-called 'moulting fluid'. The latter has subsequently been found to play an important role in the process of moulting. Thus the old cuticle, when finally shed, consists of only its outermost layers, the innermost portion having undergone dissolution while in contact with the moulting fluid (Plotnikow, 1904; Tower, 1906). Indeed, in the case of hard-bodied insects, the actual shedding of the exuviae becomes mechanically possible only after the old cuticle has been weakened in this manner (Plotnikow, 1904; Tower, 1906; Eidmann, 1924).

In addition to this primary action on the old cuticle, certain investigators have suggested that the moulting fluid serves also as a lubricant in the casting of the skin (Plotnikow, 1904; Verson, 1911; Eidmann, 1924; Hoop, 1933; Schürfeld, 1935). This function appears unlikely for, as Causard (1898) and Wigglesworth (1933, 1948*a*, *b*) point out, the fluid is ordinarily resorbed before the shedding of the exuviae takes place.

The secretion of the moulting fluid begins in synchrony with other early events in the process of moulting. Among the first of these is the detachment of the hypodermis from the overlying cuticle (Tiegs, 1922; Hoop, 1933; Wigglesworth, 1933; Kühn & Peipho, 1938; Paillot, 1939; Dennell, 1946). In species where the hypodermal cells are laced to the cuticle by cytoplasmic processes, the latter are retracted from the cuticular pore canals (Tower, 1906). The hypodermal cells elongate and generate, as it were, a surface tension in the hypodermis as a whole. In consequence, the hypodermis retracts from the overlying cuticle to form the exuvial space. The driving force almost certainly lies in the hypodermis itself, rather than in the traction of attached musculature. In fact, as Tower (1906), Kühn & Piepho (1938), and Way (1950) have observed, the sites where muscles insert on the hypodermis are the last, rather than the first regions to lose their attachments to the old cuticle. As the hypodermis retracts, the exuvial space is flooded with the moulting fluid. Meanwhile, the hypodermis begins to elaborate

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* This study was assisted by the Lalor Foundation and by a grant-in-aid from the U.S. Public Health Service.

its new cuticle whose outer boundary is, therefore, in continuous contact with the moulting fluid.

The origin of the moulting fluid has been debated in the older literature. Verson (1890, 1902, 1911) described its production by 'exuvial glands' located beneath the integument of the thorax and abdomen—a view subsequently shared by numerous investigators, including Tower (1902), Eidmann (1924), Poisson (1924), Ertogroul (1929), Wachter (1930), and Wigglesworth (1933). Yet Blunck (1923), Hoop (1933), von Buddenbrock (1930), Schürfeld (1935), Wigglesworth (1947, 1948*b*) and Way (1950) found that the dermal glands discharge their contents long after the moulting fluid has already appeared. Moreover, the glands are known to be atrophic or absent in the pupal stage of most insects, yet the moulting fluid appears promptly at the outset of adult development (Plotnikow, 1904; Schulze, 1912; Willers & Dürken, 1916; Deegener, 1928; Hoop, 1933). Thus the production of the moulting fluid by special exuvial glands seems to be a discredited theory: there is now general agreement that the principal constituents of the fluid are secretory products of the hypodermis itself.

The literature contains only scanty reference to the properties of the moulting fluid. In histological fixatives its behaviour resembles that of proteins (Tower, 1902; Plotnikow, 1904; Deegener, 1928). Schulze (1912) observed an acid reaction to litmus, Verson (1902) and Plotnikow (1904) an alkaline reaction, and Blunck (1923) and Wigglesworth (1933) a neutral reaction. Wigglesworth's (1933, 1948*b*) description of the fluid as 'salt-free' was apparently based on negative spot-tests for chloride.

The presence of enzymes in the moulting fluid has been inferred from its capacity to digest the old endocuticle (Tower, 1906; Wigglesworth, 1933, 1948*b*). For this function the minimal requirements would appear to be a proteinase and a chitinase. Why these enzymes attack the old cuticle and spare the new cuticle has been a recurrent problem (Chauvin, 1949; Richards, 1951). Hamamura, Iida & Otsuka (1940) report the presence in 'moulting fluid' of proteinase, chitinase, invertase and amylase. However, the determinations were performed on extracts of dried, pulverized, larval exuviae—a material which, in our experience, is singularly devoid of moulting fluid.

From this résumé it is evident that only limited insight into the properties of moulting fluid can be gained from a study of the literature. In view of its intimate relation to the process of moulting, the fluid merits more detailed consideration than it has received heretofore. As a first step in this direction we have studied the properties and kinetics of the moulting fluid during the pupal-adult transformation of the Cecropia silkworm.

MATERIALS AND METHODS

Pupae of the giant silkworm, *Platysamia cecropia*, were used as experimental animals. The insects were reared in large numbers and stored at 5 ± 1° C. for at least 3 months prior to use. As described by Williams (1946), such chilled pupae initiate adult development about 2 weeks after being placed at 25° C. The first visible indication of adult development is the initiation of the pupal-adult moult; i.e. the above-mentioned retraction of the hypodermis from the overlying pupal cuticle and the simultaneous appearance of the fluid-filled exuvial space. These changes occur rapidly and throughout the entire animal.

From individuals at various stages in adult development moulting fluid was withdrawn from the exuvial space for study or analysis. Each insect was anaesthetized with carbon dioxide; a disk of pupal cuticle was then excised from the tip of the abdomen, and the moulting fluid withdrawn into fine pipettes. Up to 0·1 ml. of moulting fluid could be obtained from a single individual. These procedures were performed under the dissecting microscope. Considerable care was exercised to avoid puncturing the hypodermis and thereby contaminating the colourless moulting fluid with the yellow blood. Additional methods will be described in the specific procedures reported below.

I. The Pupal Cuticle

(1) *Structure*

When a fresh fragment of mature pupal cuticle is viewed in hand-cut sections, two conspicuous layers are evident: a dark brown outer layer, the exocuticle, and a white, lamellated inner layer, the endocuticle. In histological sections a further extremely thin and inconspicuous layer is visible; namely, the epicuticle on the outermost surface of the exocuticle. In the fresh pupal cuticle the exocuticle varies in depth from 6μ in the thinnest regions to 16μ in the thickest. The depth of the endocuticle varies from 36 to 46μ. About four-fifths of the cuticle is therefore endocuticle.

In addition to these well-recognized cuticular components, we have consistently observed that a membrane suddenly appears between the old cuticle and the retracting hypodermis at the outset of the moulting process. We have been unable to discover the precise mode of origin of this membrane, though it apparently arises at the line of juncture of the cuticle and the retracting hypodermis. It is a thin, homogeneous, transparent structure, which is appressed to, but separable from, the overlying cuticle throughout the premoult period. The exuvial space and moulting fluid develop internal to the membrane. If the pupal cuticle is carefully cut away at any stage in adult development, the membrane is exposed and may be teased from the insect as a continuous sheet which extends over the entire insect including the wings. In histological sections its appearance, thickness, and staining properties resemble those of the basophilic basement membrane underlying the hypodermis. The membrane persists as a discrete structure throughout adult development, and is finally shed as an inner lining to the pupal exuviae.

A search of the literature fails to reveal any previous reference to this membrane, for which we suggest the name, 'ecdysial membrane'.* For our present purposes the ecdysial membrane is of interest because it is interposed between the moulting fluid and the old cuticle.

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* We find the ecdysial membrane to be a conspicuous structure in moulting Lepidoptera. The membrane is present, not only at the pupal-adult moult, but also at the larval-larval and larval-pupal moults. It is possible that an ecdysial membrane underlies the moulted cuticle in other Orders, and is shed as an inner lining of the exuviae.

(2) *Chemistry of the pupal cuticle*

(*a*) *The exocuticle*

The exocuticle is a complex of chitin and protein that undergoes pigmentation and 'tanning' with quinones during the first few days following the pupal moult (Pryor, 1940; Pryor, Russell & Todd, 1947). Once this sclerotization has taken place, the chitin and protein can no longer be hydrolysed by chitinase or proteinase.

(*b*) *The endocuticle*

By means of fine forceps the endocuticle was stripped from pupal cuticles and tested separately from the exocuticle for its chemical constituents. The results summarized in Table 1 confirm the presence of both chitin and protein.

Table 1. *Chemical tests on pupal endocuticle*

Test	Specificity	Reaction
Xanthoproteic	Protein; specific for aromatic groups	+
Millon's	Protein; specific for phenolic groups as in tyrosine	+
Sakaguchi	Protein; specific for arginine	+
Ninhydrin reaction on the water extract of endocuticle	Specific for α-amino-acids	+
Nitroprusside	Specific for sulphhydryl groups	0
Chitosan	Specific for chitin	+

(3) *Changes in the pupal cuticle*

Ten chilled Cecropia pupae were provided with transparent facial windows in order to observe the onset and progress of adult development (Williams, 1946); a sliver of pupal cuticle was then excised from the side of the last abdominal segment of each individual, the wound being sealed with melted paraffin. By means of a camera lucida, the thickness of the exo- and endocuticle was measured under the compound microscope in fresh hand-cut sections. In this manner the initial thickness of the cuticle was calibrated for each individual. The ten pupae were then stored at 25° C. At successive stages during the 3-week period of adult development, slices of pupal cuticle were again removed from the last abdominal segment and measured according to the above-mentioned procedure. Changes in the thickness of the cuticle were judged by comparison with the initial control measurements for each individual.

The results summarized in Table 2 show that the moulting fluid is present during 19 of the 21 days of adult development at 25° C. During the first two-thirds of this period, the overlying cuticle does not change in thickness. However, during the succeeding 6 days, the pupal endocuticle undergoes rapid erosion and dissolution, and by the end of this period has completely disappeared. Meanwhile the pupal exocuticle shows no obvious alterations. Thus, the exuviae shed by the adult moth at the time of emergence consist of the epicuticle, the exocuticle, and the ecdysial membrane.

Table 2. *Time-table for adult development of* Platysamia cecropia *at* 25° C.

(After the termination of pupal diapause)

Stage of development	Days after initiation of development	Thickness of pupal endocuticle (%)
Earliest hypodermal retraction and secretion of moulting fluid	0	100
Contours of face fully formed	2	100
Initiation of pink eye pigment; contours of genitalia fully formed but without pigment or hairs	6	100
Generalized pink eye pigment; no hairs	7	100
Generalized reddish brown eye pigment; no hairs	8	100
Complete brown eye pigment; silky hairs	9	100
Coarse white hairs; no cuticular pigmentation	11	100
Coarse white hairs and tan cuticular pigment	14	—
Face with red hairs; generalized but incomplete wing pigment	17	80
Complete wing pigment; generalized softening of pupal cuticle	18	40
Moulting-fluid resorbed	20	0
Adult emergence	21	—

That the dissolution of the endocuticle is due to the moulting fluid was demonstrated in experiments of the following type:

The cuticle of the last two abdominal segments was removed from developing animals without injury to the underlying adult tissues. A sliver of normal pupal cuticle was then implanted into the moulting fluid and the defect in the pupal cuticle capped over with a plastic cover-slip and sealed with melted paraffin. After the emergence of the hosts, the cuticular implants were removed from the exuviae, sectioned by hand, and examined microscopically.

The endocuticle had disappeared from the implants as well as from the pupal exuviae of the hosts, thus demonstrating a direct action of the moulting fluid on the pupal cuticle. This result argues in favour of the presence in the moulting fluid of a proteinase and chitinase.

II. PHYSICAL PROPERTIES OF THE MOULTING FLUID

(1) *Consistency*

When first elaborated the moulting fluid is a colourless gel resembling egg albumen in consistency. Consequently, the term, moulting *fluid*, is, at this stage, a misnomer, and one could properly speak of the moulting *gel*. This physical state persists for approximately two-thirds of adult development during which period the moulting gel has no obvious effects on the overlying pupal cuticle (Table 2). By approximately the fourteenth day, adult development has progressed to a stage signalled by the initiation of the tan pigmentation of the underlying adult cuticle. The moulting gel now changes to a moulting *fluid*, and simultaneously begins to act on the pupal endocuticle. By the twentieth day the endocuticle has disappeared leaving the unaltered exocuticle as a thin and crisp residue.

The initial gel-like character of the moulting fluid seems to be a new observation. The transition from gel to sol is of special interest since it occurs at approximately the same time that the moulting fluid begins to act on the overlying cuticle. We shall hereafter refer to the fluid during the first two-thirds of adult development as 'early-moulting fluid'; during the final one-third, as 'late-moulting fluid'.

(2) *Cellular content*

Moulting fluid at various stages in development was suspended in a hanging drop of Glaser-Locke solution. No cells were observed in the fluid at any stage. Similar treatment of blood showed the presence of numerous leucocytes.

(3) *Solubility*

The moulting fluid at all stages is completely miscible with water. Addition of ethyl alcohol caused a conspicuous precipitation in the early moulting fluid and a scanty precipitate in the late moulting fluid.

(4) *Refractive index*

Measurements of refractive index were performed in order to ascertain whether the moulting fluid might approximate a binary mixture of protein and water, since under this circumstance the index would provide a simple means of determining the protein content of the fluid. The underlying assumption proved to be false, however, so that the measurements, recorded in Table 3, fail to permit calculations of this type.

Table 3. *Properties of moulting fluid and of blood*

	Moulting fluid		Blood	
	Early	Late	Early	Late
Refractive index	1·3383	1·3377	1·3492	1·3431
H_2O content (%)	95·8 ± 0·6	94·5 ± 0·4	85	92
Total nitrogen (mg./ml.)	5·1	6·0	12·4*	9·4*
Protein nitrogen (mg./ml.)	4·7	1·1	8·3*	5·5*
Non-protein nitrogen (mg./ml.)	0·4	4·9	4·1*	3·9*
Protein concentration (g./100 ml.)†	2·96	0·71	5·2*	3·4*
pH	7·35	7·4 to 7·55	6·40	6·65
Redox potential (mV.)‡	+380 to +400	+360 to +390	+330 to +350	+306 to +308
Glucosamine (mg./ml.)	0·145	0·800	0·700	0·800
N-acetylglucosamine (mg./ml.)	0·322	2·600	0	1·200

* Unpublished data provided by William Chefurka.

† Protein nitrogen × 6·25; the assumption is made that this conversion factor is valid for insect proteins.

‡ With reference to potential of calomel electrode.

III. CHEMICAL PROPERTIES OF THE MOULTING FLUID

(1) *Water content*

Moulting fluid or blood was withdrawn from pupae at various stages in development, placed on previously weighed plastic slips, and reweighed as quickly as possible. A Roller-Smith microtorsion balance, permitting direct readings to ±0·005 mg., was used. The slips were then dried to constant weight in a desiccator.

The water content of the moulting fluid and blood was found to vary, as indicated in Table 3. It will be observed that the moulting fluid, at all stages, is more dilute than the blood, and that the transition from gel to sol is accompanied by only a minor decrease in water content.

(2) *Chemical composition*

The results of a number of qualitative tests are summarized in Table 4. The chemistry of the blood seems to remain qualitatively constant during the course of adult development. In contrast, the moulting fluid undergoes definite alterations, of which the most conspicuous are (1) a decrease in protein content to levels undetectable in the spot tests utilized; and (2) a disappearance of the enzyme tyrosinase. The positive tests for chloride, potassium and calcium are of interest in that Wigglesworth's (1933, 1948*b*) description of the moulting fluid of *Rhodnius* as 'salt free' was based on the apparent absence of chloride.

Table 4. *Chemical composition of moulting fluid and blood*

Test	Moulting fluid		Blood	
	Early	Late	Early	Late
Xanthoproteic for protein	+	0	+	+
Millon's for protein	+	0	+	+
Biuret for protein	+	0	+	+
Sakaguchi for protein	+	+	+	+
100° C. for protein	+	0	+	+
Ninhydrin for α-amino-acids	+	+	+	+
Tyrosine for tyrosinase	+	0	+	+
Catechol for tyrosinase	+	0	+	+
Fehling's for reducing sugars	+	+	+	+
Molisch's for furfural precursors	+	+	+	+
$FeCl_3 + Na_2CO_3$ for *o*-dihydroxyphenols	0	0	0	0
Nitroprusside for sulphhydryl	0	0	0	0
For chloride*	+	+	+	+
For calcium†	+	+	+	+
For potassium‡	+	+	+	+

* See Feigel (1943), p. 112.
† Wenger & Duckert (1948), p. 215.
‡ Wenger & Duckert (1948), pp. 231–2.

(3) *Total nitrogen content*

Moulting fluid was analysed for total nitrogen by the micromethod of Umbreit, Burris & Stauffer (1949). Samples consisting of 0·2 ml. moulting fluid were diluted to 10 ml. with water, and tests performed on 0·5 ml. aliquots.

Results recorded in Table 3 show that the total nitrogen of moulting fluid increases from 0·51 % in early development to approximately 0·60 % in late development. This is equivalent to an absolute increase of 0·9 mg. nitrogen per ml. of moulting fluid. The corresponding values for blood, according to the detailed but unpublished measurements of William Chefurka, are 1·2 and 0·9 % nitrogen.

(4) *Protein content*

The proteins of moulting fluid were precipitated with trichloracetic acid, centrifuged, digested, and analysed for nitrogen by the Conway microdiffusion technique (1947); the titrations were performed with a microburette described by Black (1949).

The results of a total of eight analyses are summarized in Table 3. It is evident that the protein content of moulting fluid decreases markedly during the course of adult development; in late moulting fluid the concentration was one-fourth that of early fluid. Evidently, in the early fluid, over 90% of the total nitrogen is in protein; in the late fluid less than 20% is in protein. Blood at all stages contains a higher concentration of protein than the moulting fluid (Table 3).

(5) *pH*

The pH of moulting fluid and blood was measured with a Beckman pH meter in conjunction with a one-drop glass electrode and a small calomel electrode. Results summarized in Table 3 show that at all stages the moulting fluid is slightly alkaline, and that the hydrogen-ion concentration in the blood is nearly ten times that in the moulting fluid.

(6) *Oxidation reduction potential*

The redox-potential of moulting fluid was examined because pronounced changes in potential during the course of development might activate enzymes that had been held inactive or render some component of the endocuticle available to enzymes already present.

The cuticle of the last two abdominal segments was removed from unanaesthetized developing pupae, and standardized microplatinum and calomel electrodes inserted directly into the moulting fluid. In determinations performed on blood, a few drops were removed with fine pipettes, dispensed into small vessels, and promptly measured.

Results obtained with the Beckman instrument and recorded in Table 3 show that the moulting fluid possesses a slightly higher potential than the blood, and that only minor alterations occur during the course of adult development.

IV. ENZYMATIC ACTIVITY OF THE MOULTING FLUID

(1) *Chitinase*

As far as is known, the chitinases are the only biological reagent capable of hydrolysing the polymer, chitin, into its monomer, *N*-acetylglucosamine. Since the chitin-containing endocuticle undergoes dissolution in the presence of the moulting fluid, a detailed study was performed of the chitinase activity of moulting fluid.

(*a*) N-*acetylglucosamine and glucosamine*

Moulting fluid and blood were first assayed for the products of the chitinase reaction; namely, *N*-acetylglucosamine and the deacetylated derivative, glucosamine.

0·2 ml. of moulting fluid or of blood was diluted with 1·0 ml. of phosphate buffer, pH 7·1; the glucosamine content of 0·5 ml. aliquots was then assayed by the colorimetric procedure of Elson & Morgan (1933). Since this test is insensitive to *N*-acetylglucosamine, and to partial breakdown products of chitin, the latter were hydrolysed to glucosamine using 4 N-HCL, according to the method of Palmer, Smyth & Meyer (1937). The solutions were brought to pH 6·0–6·4 with 6 N-NaOH, diluted to 5, 10, or 25 ml. and then re-analysed for glucosamine. The analytical procedure was accurate to within ±5%.

The results, recorded in Table 3, show that both glucosamine and *N*-acetylglucosamine are present in the moulting fluid at all stages. During the period of destruction of the old endocuticle, their concentrations in the moulting fluid increase five- and eightfold, respectively, and detectable concentrations of *N*-acetylglucosamine appear for the first time in the blood. The recruitment of *N*-acetylglucosamine, over and above its concentration in the blood, is strong evidence that the late-moulting fluid contains a chitinase.

(*b*) *Chitinase activity*

Tests for chitinase were performed on purified crayfish chitin most kindly provided by Prof. A. Glenn Richards and on crayfish chitin reprecipitated by the method of Karrer & Hofmann (1929). Both materials showed negative reactions when tested for proteins and reducing sugars; when hydrolysed with 12 N-hydrochloric acid, the yield of glucosamine conformed to theory.

0·1 or 0·2 ml. of moulting fluid was diluted with 1 ml. of phosphate buffer at pH 7·1 and added to tubes containing weighed amounts of chitin. In control preparations the moulting fluid was inactivated by boiling. Parallel tests were performed on the insect blood. The tubes were incubated for 5 days at 37·5° C., their contents filtered, and assayed for glucosamine and *N*-acetylglucosamine by the technique described above.

Each sample was corrected for the amount of glucosamine and *N*-acetylglucosamine initially present in the moulting fluid, as determined in control experiments utilizing heated moulting fluid. The difference, representing *in vitro* formation of the breakdown products, was taken as a measure of chitinase activity.

The results recorded in Table 5 are typical of a series of experiments of this type. Chitinase activity was in all cases much higher in late than in early moulting fluid. In the experiments described in Table 5, the difference was 13½-fold.

Table 5. *Chitinase activity of early and late moulting fluid when incubated 5 days with chitin*

Stage of donor	Chitin substrate (mg.)	Vol. moulting fluid added (ml.)	Glucosamine + *N*-acetylglucosamine (mg.)		
			Unheated moulting fluid	Heated moulting fluid	Difference
Early (5th to 11th day)	6·7	0·2	0·126	—	0·040
	8·3	0·2	0·140	—	0·054
	5·4	0·2	—	0·086	—
Late (12th to 19th day)	8·9	0·2	1·360	—	0·560
	11·0	0·2	1·520	—	0·720
	10·4	0·2	—	0·800	—

When a single sample of moulting fluid was tested in graded dilutions, the chitinase activity was found to be proportional to the concentration of moulting fluid. In all these procedures the enzyme appeared to be remarkably stable. Thus, when the incubation period was extended from 5 to 11 days, the final concentration of breakdown products was greatly enhanced. Parallel tests on the insect's blood failed to reveal any chitinase activity at any stage in adult development.

(2) *Proteinase*

The proteinase activity of moulting fluid was measured in terms of its hydrolytic action on gelatin.

(*a*) *Viscosity measurements*

A series of Ostwald 5 ml. viscosimeters was first calibrated at 37° C. with distilled water and with a standard 2% solution of Difco gelatin. 5 ml. of gelatin were then placed in each of a series of tubes, along with a crystal of thymol to prevent bacterial growth. The temperature was held constant at 37° C. by the use of a water-bath.

A precise volume of moulting fluid was then added to each tube, mixed with the gelatin, and measurements of the latter's viscosity begun immediately. The efflux time was recorded at frequent intervals until constancy was observed. Parallel experiments were performed on the insect's blood and on moulting fluid which had previously been heated and centrifuged. Comparisons were also made with the action of trypsin on the test system.

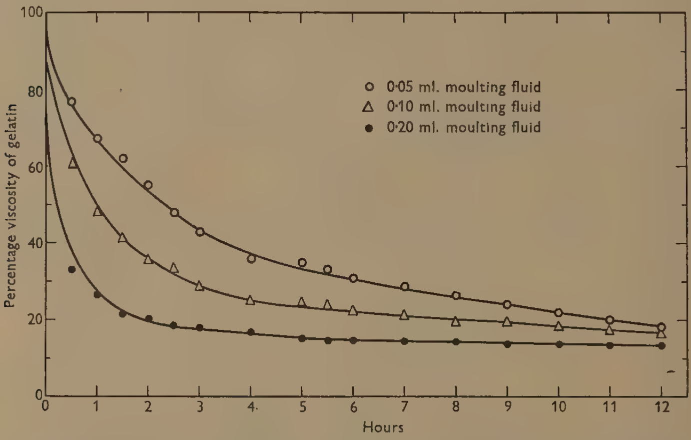

Fig. 1. Percentage change in the viscosity of a standard gelatin solution when incubated with late moulting fluid. On the vertical axis 0% corresponds to the viscosity of distilled water; 100%, to the initial viscosity of the gelatin solution.

Early moulting fluid, obtained from animals during the first 2 weeks of adult development, had no significant effects on the viscosity of the gelatin. In contrast, the late moulting fluid, obtained from animals during the third week of adult development, showed considerable proteolytic activity.

A typical experiment is recorded in Fig. 1, where it will be observed that the late moulting fluid decreased the viscosity of gelatin, over a period of 12 hr., to a value within 20% of that of distilled water. The rate of change in viscosity was proportional to the concentration of moulting fluid. 0·2 ml. of the moulting fluid showed approximately the same proteinase activity as 0·3 ml. of 0·45% trypsin.

Late moulting fluid, previously heated to 100° C., was without significant effects after incubation with gelatin for 20 hr. Negative results were also obtained with the insect's blood at all stages in adult development.

(*b*) *Formol titrations*

At the end of each series of viscosity measurements the degree of degradation of the gelatin was determined by formol titration (Koch & Hanke, 1948). These measurements, when suitably corrected for the free amino groups initially present in the gelatin and the moulting fluid, confirmed the proteinase activity of the late moulting fluid.

V. RESORPTION OF THE MOULTING FLUID

As noted in Table 2, the moulting fluid is resorbed during the final 2 days of adult development. Consequently, the surface of the adult moth at the time of emergence is only superficially moist. In experiments in which the disappearance of the fluid was witnessed through plastic windows in the pupal cuticle, the antennal 'cases' of the pupa were found to be the first sites to empty of fluid.

Resorption proceeds at all levels of the insect. The exit of the moulting fluid is accompanied by the entrance of air; the latter first appears in the antennal 'cases' and gradually fills the entire exuvial space. The fluid obviously disappears into the underlying insect, apparently through any region of the integument with which it is in contact. Drops of moulting fluid, trapped between a plastic window and the cuticle of the underlying insect, appeared to be resorbed *in situ*, regardless of their position. Moreover, the fluid underwent normal resorption when isolated anterior or posterior ends of pupae were caused to metamorphose. The ability to resorb the moulting fluid is, evidently, a generalized property of the newly formed integument—presumably of the hypodermis itself.

This conclusion is in agreement with that of Wigglesworth (1933) and suggests, in turn, that the moulting fluid may have a more dynamic relation to the insect than has been demonstrated heretofore. This possibility was tested in the following manner:

The pupal cuticle was removed from the last two abdominal segments of a series of previously chilled pupae, and the opening sealed to a plastic slip by means of melted paraffin. After the insects had initiated adult development, each pupa was anaesthetized with carbon dioxide and the plastic slip carefully removed. To the moulting fluid 0·1 ml. of glycine solution was then added containing 10 μg. of ^{14}C carboxyl-labelled-DL-glycine (activity of 4052·7 CPM) and 40 μg. of unlabelled DL-glycine. The opening in each animal was finally resealed with a plastic slip.

At specific intervals thereafter, animals were sacrificed and assayed for radioactive protein. In this procedure the pupal cuticle was removed and the moulting fluid washed away in a stream of distilled water. The insect was then homogenized in distilled water to a total volume of 5·0 ml. An equal volume of 10% trichloracetic acid was added to precipitate the protein, the homogenization repeated, and the protein collected by centrifugation. The protein was then washed twice on the centrifuge in 5% trichloracetic acid, twice in methyl alcohol, and twice in ether. The final protein fraction was thoroughly dried, layered in 'infinite thickness' on weighed disks, and assayed for radioactivity by means of a gas-flow Geiger counter.*

The results recorded in Fig. 2 show that the tagged amino-acid promptly entered the organism and was incorporated into the proteins of the moth. The adult protein showed maximal radioactivity on the second day following injection, and subsequently underwent a decrease. The latter we attribute, in part, to the metabolism of the protein and to the resulting loss of ^{14}C as carbon dioxide.

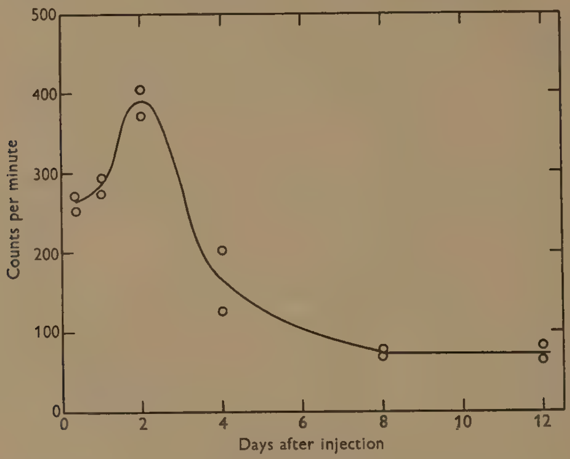

Fig. 2. Radioactivity of proteins extracted from developing Cecropia after the injection of ^{14}C-labelled DL-glycine into the moulting fluid of animals on the second day of adult development.

DISCUSSION

As the experimental results indicate, the moulting fluid at all stages shows physical, chemical and enzymatic properties which differ quantitatively and, in several respects, qualitatively from those of the blood. The moulting fluid is therefore a distinctive fluid compartment of the moulting insect—as distinctive as the blood itself. The insect is 'immersed' in this special fluid during the premoult period: all integumentary surfaces are bathed by the moulting fluid. Consequently, the status of the organism at this time is not unlike that of an aquatic insect breathing through spiracles at the water surface.

The moulting fluid, as we have seen, is secreted at the outset of the moulting process. As the hypodermis detaches itself from the pupal cuticle and retracts, the intervening space is simultaneously flooded with moulting fluid. As Hinton (1946, 1948) has emphasized, this detachment and retraction of the hypodermis is the real

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* We wish to thank Dr Donald Buchanan of the Argonne National Laboratory for assistance in these experiments.

moult; it is merely a technicality that the old discarded cuticle continues to be present for a period thereafter.*

As considered in the Introduction, all the evidence points to the hypodermis as the source of at least the principal constituents of the moulting fluid. Thus the hypodermis, while engaged in secreting and organizing the new cuticle, is called upon to perform yet another task, the synthesis and secretion of the moulting fluid.

More or less abrupt changes in the moulting fluid take place during the course of adult development. The early moulting fluid, present during the first two-thirds of adult development, is a gel with no demonstrable proteolytic activity and only a trace of chitinase activity. The late moulting fluid, present during the final third of adult development, is a sol with considerable enzymatic activity. The obvious function of the early fluid is to protect the delicate underlying insect—a role analogous to that of the amniotic fluid of the mammal. The late moulting fluid is an agent for dissolving the bulk of the old cuticle and permitting the escape of the mature insect.

Most of the observed differences between early and late fluids appear to be secondary to the striking increase in the fluid's enzymatic activity on about the fourteenth day of adult development. The action of the proteinase on the old endocuticle, and on the proteins of the moulting fluid itself, can account for the following changes in the fluid: the gel–sol transformation, the decrease in protein nitrogen, the increase in non-protein nitrogen, and the slight increase in pH. Indeed, all of these changes are encouraged when a pure proteinase, such as trypsin, is incubated with a gelatin solution. The action of chitinase on the old endocuticle is obviously responsible for the considerable increase in glucosamine and *N*-acetylglucosamine; these compounds, in turn, contribute to the increase in non-protein nitrogen.

Acting jointly on the overlying cuticle, the chitinase and proteinase of the late fluid lead to the hydrolytic breakdown of the old endocuticle. But the old, sclerotized exocuticle is spared by virtue of the innate resistance to enzyme action of its tanned protein-chitin complex. The enzymatic reactions reduce the thickness of the old cuticle by approximately 80%, corresponding to the depth of the endocuticle. Indeed, the reduction is almost 100% along the predetermined 'rupture' or ecdysial lines where the exocuticle is extremely thin or absent. Meanwhile, as demonstrated in the tracer studies, the breakdown products of the old endocuticle are continuously reclaimed from the moulting fluid and utilized by the adult moth.

The mechanism for the total resorption of the moulting fluid during the final 2 days of development is unknown. The resorption, as we have seen, appears to take place through the integument as a whole. Provided that the integument is permeable to water, a passive process such as osmosis would favour the resorption of the fluid, since the osmotic pressure of the blood is apparently much higher than that of the moulting fluid. Under this point of view, the final resorption would signal the end of a pre-existing steady state in which active secretion had continuously counterbalanced osmotic resorption. Yet we find that precocious resorption of the moulting fluid fails to occur in animals placed at 5° C.; that is, under conditions greatly inhibiting the active metabolic process while only slightly impairing the passive osmotic process. It is clear that the resorption of the moulting fluid is poorly understood and merits further study.

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* A tardy appreciation of this fact has led to considerable confusion in the literature, especially with respect to the pupal-adult transformation. Thus it is commonly assumed that if one looks inside a 'pupa', one encounters an adult moth more or less fully formed. But, in point of fact, such a finding gives assurance that the pupal stage ended days, weeks, or months earlier, when the hypodermis retracted from the pupal cuticle and began to secrete the adult cuticle. Thereafter, one is dealing not with a pupa but with a developing adult. In short, the stage of an insect is not always obvious on external examination and can only be identified with certainty in terms of the type of cuticle to which the hypodermis is attached.

Finally, there remains to be considered the basis for the moulting fluid's selective action on the overlying old cuticle while the underlying new cuticle is spared. In connexion with this classical problem, it has not previously been appreciated that the early moulting fluid is extremely low in enzymatic activity. Thus, in the case of the Cecropia silkworm, the new cuticle completes two-thirds of its development in contact with moulting fluid incapable of digesting protein. Moreover, we have observed that the enzymatic activity of the moulting fluid rapidly increases in synchrony with the sclerotization of the underlying new exocuticle—a tanning of the chitin protein complex sufficient, in itself, to account for its resistance to enzymatic attack.

Further insight into these events was obtained in endocrinological experiments in which Cecropia pupae were caused to undergo a second pupal moult by the implantation of active corpora allata (Williams, 1952). In such preparations the new pupal cuticle underwent precocious pigmentation and sclerotization in certain localized regions, while other regions remained unsclerotized. It is of particular interest and importance that the moulting fluid became enzymatically active, and attacked the old cuticle in precisely those zones overlying the precociously sclerotized new cuticle.

Experiments of this type suggest a causal relation between the sclerotization of the new cuticle and the activation of enzymes in the overlying moulting fluid. Perhaps the secretion of enzymes into the moulting fluid is coupled to the secretion of the dihydroxyphenol or the quinone which tans the exocuticle. Alternatively, the early moulting fluid may already contain precursors of proteinase and chitinase which are activated by some by-product of the sclerotization reaction. Though the present study failed to reveal any such pro-enzymes in early moulting fluid, it is worth recalling that all extracellular proteinases, thus far characterized, are secreted as pro-enzymes.

SUMMARY

1. The initiation of adult development in the pupa of the Cecropia silkworm is accompanied by a retraction of the hypodermis from the pupal cuticle; the exuvial space is simultaneously flooded with the moulting fluid.

2. During the first two-thirds of adult development the moulting fluid is a dilute, aqueous, proteinaceous gel resembling egg albumen; at this time it is without effects on the pupal cuticle.

3. On approximately the fourteenth day of adult development the gel is converted into a sol and shows a considerable increase in chitinase activity and the first demonstrable proteolytic activity.

4. The late, active moulting fluid then begins to hydrolyse the protein and chitin in the overlying pupal endocuticle.

5. By the twentieth day the old endocuticle has disappeared. The tanned protein-chitin complex of the sclerotized exocuticle is not attacked.

6. Finally, the moulting fluid is completely resorbed into the underlying insect. The residual pupal exocuticle forms the bulk of the exuviae.

7. Moulting fluid and blood are compared in respect to the following properties: consistency, cellular content, solubility, refractive index, water content, chemical composition, total nitrogen, protein nitrogen, pH, redox potential, and enzymatic content.

8. The moulting fluid shows numerous quantitative and qualitative differences from the blood. The fluid is, evidently, a distinctive secretory product of the underlying integument—presumably of the hypodermis itself.

9. A dynamic state exists between the moulting fluid and the underlying insect. Radioactive glycine, injected into the moulting fluid, was promptly resorbed and incorporated into the protein of the adult moth.

10. Several lines of evidence suggest that the secretion or activation of the moulting fluid enzymes are synchronized with the sclerotization of the underlying new cuticle. This synchronization, it is suggested, may serve to protect the new cuticle from the hydrolytic action of the moulting fluid.

Mr Dietrich Bodenstein, Dr Leigh E. Chadwick, Prof. A. Glenn Richards, and Dr Berta Scharrer were most helpful in reading a preliminary manuscript of this paper.

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THE LIPIDS OF SEA-URCHIN SEMEN

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(*Received* 13 *March* 1953)

The constitution of the lipid material present in semen has received very scant attention. No reference appears in the literature relating to the component fatty acids of semen lipids, although the presence of neutral fat, free fatty acid, free and combined sterols, phosphatides, and sphingomyelin has been reported by Woodhouse (1928) in the sex organs of domestic animals and of humans; likewise, Sakaki (1913) reported the presence of phosphatides and sphingomyelin in the human placenta; Sano (1922) reported the presence of these phosphatides in the fresh 'sperm freed from membranes and connective tissue' of the cod, porgy and salmon. More recently, Lardy & Phillips (1941), in an investigation of the sources of energy for bull spermatozoa, showed that the phospholipid content of both semen and spermatozoa decreased when incubation was carried out in the absence of extracellular substrates but in the presence of oxygen.

Our knowledge of the physiology of sea-urchin spermatozoa is primarily due to Rothschild and co-workers who have shown (see Rothschild, 1951) that sea-urchin spermatozoa differ from those of mammals, in which the energy for motion is provided from outside the spermatozoa in the form of substrates in the seminal plasma. The energy necessary for the movement of sea-urchin spermatozoa does not arise to any great extent from enzymatic breakdown of substrates in their seminal plasma since they are capable of prolonged activity when suspended in pure sea water. Serious doubt has been cast on the view that the energy required is derived from breakdown of intracellular carbohydrate (Rothschild & Cleland, 1952). These authors have shown that the phospholipid content of the spermatozoa of *Echinus esculentus* amounts to about 5·5 % of the dry weight of the spermatozoon, while the seminal plasma contains much less. On aerobic incubation of the semen diluted with sea water the phospholipid content was shown to decrease, indicating that oxidative breakdown of the phospholipid material was the principal source of energy required.

It was considered that further information concerning the physiology of sea-urchin spermatozoa might be derived from a more detailed study of sea-urchin semen lipids.

METHODS AND RESULTS

1193 g. of the semen of *E. esculentus* kindly placed at our disposal by Lord Rothschild was extracted by refluxing with acetone. The solid tissue was pressed and re-extracted with acetone, this process being repeated three times, followed by a final extraction of the tissue with light petroleum (b.p. 40/60). The acetone extracts were combined, acetone distilled off and the residue thoroughly extracted with light petroleum to which was added the final petroleum extract of the tissue. After removal of the petroleum, the lipid extract was heated at 100° C. under reduced pressure for a short time to remove the last traces of solvents and moisture, when 11·40 g. (0·95 %) of lipids were obtained.

The phospholipids were separated from the total lipids by crystallization from acetone at 0° C. (4 ml./g.), the insoluble fraction then being freed from other entrained lipids by three further crystallizations from acetone at the same temperature and dilution. In this way the total lipids were resolved into 8·440 g. (74·0 %) non-phosphorus-containing lipids and 2·960 g. (26·0 %) phospholipids, the nitrogen (Kjeldahl) and phosphorus (molybdate) contents of the latter fraction being 2·0 and 3·2 % respectively, giving an atomic ratio N : P of 1·4 : 1.

Table 1. *Sea-urchin semen lipids*

	Non-phospholipid 'glycerides'	Non-phospholipid 'glycerides' fatty acids excluding unsaponifiable	Phospholipid fatty acids
Saponification equivalent	415·1	288·1	286·1
Iodine value	199·5	246·6	189·8
Free fatty acids (as % oleic)	44·5	—	—
Unsaponifiable (wt. %) Sterols	12·4	—	—
Non-Sterols	24·7	—	—
Neutral fat	18·4	—	—

After determining the characteristics of the non-phosphorus-containing lipids the fraction was saponified and unsaponifiable material (2·960 g. iodine value 144·0) extracted from the soaps. Crystallization of the unsaponifiable material from light petroleum gave rosette form crystals. These were recrystallized from the same solvent, both mother liquors being combined and the petrol removed, the unsaponifiable material in this way being resolved into two fractions. The sterol content of each fraction was determined by the method described by Popjak (1943), the crystalline fraction containing 28 %, that of the fraction recovered from the mother liquors containing 38 %, the mean sterol content of the unsaponifiable material being 34 %.

In Table 1 are summarized the data obtained from the separation and resolution of the lipids, together with the characteristics of the non-phosphorus-containing lipids and phospholipid fractions respectively.

From the above data it can be deduced that the semen lipids of *E. esculentus* consisted of 13·6 % neutral fat, 32·9 % free fatty acid, 26·0 % phospholipids, 9·2 % sterols, 18·3 % other unsaponifiable material.

Component fatty acids of the non-phosphorus containing lipids. 4·95 g. mixed fatty acids (recovered from the soaps after extraction of the unsaponifiable material and comprising the free acids, together with those derived from the neutral glycerides) were resolved by crystallization from acetone (10 ml./g.) at −60° C. into two

TABLE 2. *Crystallization of sea-urchin mixed fatty acids*

Fraction	Conditions	Weight		Iodine value
		g.	% of total	
A	Insoluble in acetone at −60° C.	1·40	28·3	75·8
B	Soluble in acetone at −60° C.	3·55	71·7	296·6

Table 3. *Fractionation data for sea-urchin ester fractions*

Fraction	Weight (g.)	Saponification equivalent	Iodine value
	Methyl esters of acids A		
A1	0·55	283·1	37·5
A2	0·57	315·2	82·7
A3[*]	0·19	—	—
Total	1·31	—	—
	Methyl esters of acids B		
B1	0·60	288·1	216·6
B2	0·57	301·2	261·9
B3	0·58	310·2	284·1
B4	0·60	311·0	312·5
B5	0·59	317·4	321·6
B6	0·56	345·4[†]	294·0
Total	3·50	—	—

[*] A3 unsaponifiable matter (by weight) 0·05 g. Remaining esters calculated as sap. equiv. 315·2, iodine value 82·7 (A2).

[†] Equivalent of B6 esters freed from unsaponifiable matter 340·3. Unsaponifiable matter (by weight) 0·03 g.

Spectroscopic analysis of acids recovered from A1 and A2:

$E^{1\%}_{1\,\text{cm.}}$	234 mμ	268 mμ	301 mμ	315 mμ
A1 (unisomerized)	12·2	4·1	—	—
A1 (isomerized)	46·2	52·0	15·2	14·6
A2 (unisomerized)	19·4	6·0	—	—
A2 (isomerized)	90·4	113·4	32·1	31·9

Table 4. *Sea-urchin semen component fatty acids*

Acid	A (28·3 %)[*] (%)[†]	B (71·7 %)[*] (%)[†]	Total fatty acids excluding unsaponifiable	
			% (w/w)	% by mol.
Palmitic	35·0	—	10·1	11·5
Unsaturated C_{16}	—	2·8 (−3·0)	2·1 (−3·0)	2·4
Unsaturated C_{18}	19·0 (−2·5)	34·2 (−5·4)	30·4 (−4·9)	31·9
Unsaturated C_{20}	30·5 (−2·5)	49·8 (−8·1)	45·1 (−7·0)	43·4
Unsaturated C_{22}	11·5 (−2·5)	12·3 (−8·0)	12·3 (−6·5)	10·8
Unsaponifiable	4·0	0·9	—	—

[*] Groups as % (w/w) of total fat.

[†] Component acids as % w/w of group.

Figures in brackets denote mean unsaturation of each group of acids (Hilditch, 1947, p. 23).

fractions, consisting of mainly saturated and monoethenoid acids, and mainly polyethenoid acids respectively.

Each group of acids was methylated (Bjarnason & Meara, 1944), fractionated, and from the analytical data obtained the composition of each ester fraction was calculated (Hilditch, 1947, pp. 498–570), and therefrom the component acids in each group of acids, and ultimately the component fatty acids of the unsaponifiable free non-phosphorus-containing lipids.

DISCUSSION

If it can be assumed that the seminal plasma of sea-urchin semen is as deficient in non-phosphorus-containing lipids as it is in phospholipids, then the present investigation gives some indication of the nature of the lipids of sea-urchin spermatozoa.

The component fatty acids of the non-phospholipid fraction indicate that this portion of the total lipids conforms to the general character of marine oils by virtue of the low saturated acid content and high content of highly unsaturated acids of the C_{18}, C_{20} and C_{22} series. This is seen by comparing sea-urchin semen fatty acids with those of the lipids from two other invertebrate marine animals, the mussel (*Mytilus edulis*) (Lovern, 1938), and the prawn *Leander serratis* (Klem, 1935) (Table 5).

The content of unsaturated C_{16} acids in the sea-urchin semen mixed fatty acids is somewhat lower, and the unsaturated C_{20} acids content correspondingly higher than those of the other two invertebrate fats recorded, this relationship holding for the depot fats and liver lipids of most marine animals. Although for obvious reasons no rigid comparison can be made between sea-urchin semen fatty acids and those present in the fats from different organs of other marine organisms, nevertheless, there is sufficient resemblance between these fats to indicate that the composition of the fatty acids derived from the sea-urchin semen lipids is typically marine in type, containing a characteristically low amount of saturated acids and a high proportion of highly unsaturated acids of the C_{18}, C_{20} and C_{22} series. The possibility that these unsaturated fatty acids may play some role in the fertilization reaction has been discussed by Rothschild (1952).

No explanation can as yet be given to account for the high free acidity of the semen fat. It is probable that this is due to lipolytic hydrolysis of part of the original neutral fat and possibly of sterol esters having proceeded to a considerable extent. On the other hand, it has often been observed in these laboratories that lipids extracted from fresh livers, where fat is being actively metabolized, have a relatively high free acidity, and it is possible that a somewhat similar process is operating in sea-urchin spermatozoa. A further observation is that high free acidity appears to be concomitant with a relatively high unsaponifiable (and therefore sterol) content, this tendency also being observed in the liver lipids of the ox, sheep and pig (Hilditch & Shorland, 1937) and of the elephant (Cama, 1952). In the present investigation it was not found possible to determine whether the sterols occurred free or combined, but if the sterols of sea-urchin spermatozoa resemble those found in other marine organisms it is probable that the bulk occurs as free sterols.

The high content of phospholipids (26 %) in sea-urchin semen lipids is of the same order as that found (40 %) in the lipids of the common mussel. This phospholipid content, being of the order of 2·3 % calculated on a dry weight basis appears to be in keeping with the value of 5·5 obtained by Rothschild & Cleland (1952), indicating that about 60 % of the phospholipid content has been metabolized in the interval between collecting the semen and its extraction. It is not possible to say, however, whether any of the non-phosphorus-containing lipids have also been metabolized.

Table 5. *Component fatty acids of aquatic invertebrate animal lipids* (%, *w*/*w*)

	Sea-urchin semen non-phospholipid 'glycerides'	Common mussel non-phospholipid 'glycerides'	Prawn total lipids
Myristic	10·1	1·9	1·5
Palmitic		16·7	9·5
Stearic		1·7	2·0
Unsaturated C_{16}	2·1 (−3·0)	10·9 (−2·5)	13·5 (−2·0)
Unsaturated C_{18}	30·4 (−4·9)	21·5 (−4·1)	32·0 (−3·3)
Unsaturated C_{20}	45·1 (−7·0)	29·9 (−7·3)	34·0 (−6·0)
Unsaturated C_{22}	12·3 (−6·5)	13·9 (−9·3)	7·0 (−10·0)
Unsaturated C_{24}	—	3·5 (−?)	—

The N : P ratio of the crude phospholipids gives some indication as to the nature of their constituents. This ratio is of the order of 1 : 1 for vegetable phospholipids but of the order of 1·5 : 1 for phospholipids of animal origin. The ratio in the case of the sea-urchin semen phosphatides, being 1·4 : 1, is in agreement with this value and indicates the presence of monoaminophosphatides, together with a certain amount of diaminophosphatides, either sphingomyelin or related substances (which are known to occur in sex organs, the placenta and fish sperm), contaminated possibly with smaller amounts of non-phosphorus-containing lipoproteins.

From the characteristics of the mixed fatty acids recovered from the phospholipids, sapon. equiv. 286·1, iodine value 189·9, it is seen that these acids also can be considered to be normal for those derived from the phospholipids of marine species.

It appears, therefore, that there are adequate reserves of lipid material, both glyceridic and possibly in the form of free fatty acids, in addition to phospholipids, to provide a considerable amount of energy for the motility of sea-urchin spermatozoa when suspended in sea water.

SUMMARY

1. The specimen of sea-urchin semen examined contained 0·95 % lipid material consisting of 13·6 % neutral fat, 32·9 % free fatty acids, 26·0 % phospholipids, 9·2 % sterols and 18·3 % other unsaponifiable material.

2. The component fatty acids of the non-phosphorus-containing lipids have been computed to be: palmitic 10·1, unsaturated C_{16} 2·1 (−3·0), C_{18} 30·4 (−4·9), C_{20} 45·1 (−7·0), C_{22} 12·3 (−6·5) %, w/w, this being a composition which can be regarded as typical of a marine animal fat.

3. The N : P ratio of the phospholipids indicated the presence of mono- and diaminophosphatides and possibly the presence of non-phosphorus-containing lipoproteins.

4. Adequate reserves of non-phosphorus-containing lipids are present in the spermatozoa, in addition to phospholipids to serve as a source of energy required for movement.

We are greatly indebted to Lord Rothschild, F.R.S., for the provision of the specimen of sea-urchin semen which made the investigation possible, and to Professor T. P. Hilditch, F.R.S., for his interest in this work.

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OSMOTIC AND IONIC REGULATION IN THE ISOPOD CRUSTACEAN *LIGIA OCEANICA*

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(*Received* 20 *January* 1953)

I. INTRODUCTION

Little is known of the physiology of isopods, and although much work has been done on the osmoregulation of other crustacean groups in both marine and fresh-water environments, the isopods have been rarely studied. The occurrence of related isopod species in marine, fresh-water and terrestrial habitats should provide interesting material for comparative physiological study. *Ligia oceanica* (L.) is the largest of the endemic species and so a suitable subject for experimental work. The recent development of a micro-freezing-point determination of osmotic pressure (Ramsay, 1949) and the micro-analytical techniques of Robertson & Webb (1939) make possible the analysis of the small quantities of blood obtainable from these animals.

This work was begun in order to find out if *L. oceanica* could control the concentration of salts and water in the blood in aquatic and terrestrial conditions. To this end the osmotic pressure of the blood was measured in animals living in varied conditions of salinity and oxygenation, and in moist and dry terrestrial conditions. The influence of moulting on the concentration of salts in the blood was also followed, but without any clear-cut results.

Previous measurements of the osmotic pressure of the blood in various isopod species have been made. Widmann (1935), using a cryoscopic method, gives a range of 2·2–2·5Δ° C. for normal *L. oceanica* collected from the shore; Bateman (1933) with vapour pressure determinations in the same species living on moist seaweed gives Δ2·29° C. (recalculated from the mean molar concentration given). Blood calcium was determined in the related *L. exotica* by Numanoi, who found 0·11 mg. calcium in 0·1 ml. blood (1934) and 0·97 mg. ± 0·06 in 1 ml. blood (1937). Of this total calcium, 75 % was found to be dialysable and 25 % bound to proteins.

II. METHODS

Experimental animals were kept in standard conditions of sand wet with sea water (from Plymouth, *c.* Δ1·98° C.) and were fed with seaweed (*Fucus* spp.). During experiments they were kept at room temperatures (18–20° C.). Osmotic changes could be followed in single animals as only 0·001 ml. or less of blood was required for the freezing-point determination; successive sampling had no measurable effect on the animal or on the freezing-point of the blood. Blood was drawn from the heart at the back of the thorax by a fine glass cannula. The small wound appeared to heal rapidly and there was little bleeding from the point of injury. For freezing-point measurements, animals were often used more than once; but when much larger blood samples were drawn for inorganic analysis, the animals were only used once, although they appeared to recover when replaced in suitable conditions.

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Freezing-point measurements were all made in duplicate, the individual figures given in this paper being the means. The results are given as Δ° C. which may be converted into % NaCl concentrations by using the relationship Δ/NaCl (w/v) = 0·60 (Ramsay, 1949).

Inorganic analyses of the common ions were made by the following methods. All samples for cation determinations were first dried and then ashed with added sulphuric acid at 450° C. to remove organic matter. Sodium was precipitated and weighed as sodium zinc uranyl acetate; potassium was precipitated as potassium silver cobaltinitrite and then titrated with ceric sulphate; calcium was twice precipitated as oxalate and titrated with ceric sulphate. (These three methods were those of Robertson & Webb, 1939.) Magnesium was precipitated with hydroxyquinoline, the precipitate brominated and titrated iodometrically (Cruess-Callaghan 1935). Sulphate was precipitated with benzidine and the red colour (maximum absorption at 450 μ) obtained by treatment with sodium β-naphthoquinone-4-sulphonate was compared with standard sulphate solutions in a Spekker photometer (Letonoff & Reinhold, 1936). Chloride was replaced by iodate which was determined iodometrically (Sendroy, 1937; Van Slyke & Hiller, 1947). Later analyses of chloride were made by Conway's microdiffusion technique (Conway, 1950, p. 184). This latter method gave results somewhat higher than the former. Total base was determined by converting bases to sulphate and measuring this gravimetrically as benzidine sulphate (Hald, 1933, 1934).

With the small quantities of blood available from single animals (0·1–0·5 ml.) the accuracy of the results obtained by these analytical methods was not so great as described in the original papers. Standard errors of the mean values were within 2·8 % for sodium, calcium and sulphate, 7·8 % for magnesium, 17 % for potassium, and 3 and 7 % for chloride depending on the method used. In view of the variations of Δ_{blood} from one individual to another, it seems likely that a large part of the observed variation in ion concentrations arises from a variation in the animals rather than from errors of analysis.

The volume of the blood samples was measured by weighing an equal quantity of distilled water in the same capillary pipette. Samples could not be weighed directly as the blood coagulated very rapidly. Concentrations of ions are expressed as mg./ml. blood and as m.equiv./l. The average water content was 92·65 % of the weight, about 954 mg./ml. (Specific gravity is assumed to be about 1·030.)

III. EXPERIMENTAL RESULTS

Osmotic pressure of the blood in standard conditions

Blood from animals in the conditions defined as standard had a freezing-point depression between Δ 1·983 and Δ 2·327° C. (mean Δ 2·15° ± 0·04 s.e., $n = 24$). In natural circumstances the variation within a population may be greater than this, as the conditions in which the animals live are less rigorously defined. The mean value of Δ2·15° C. is greater than that normally observed in marine invertebrates, where the blood is usually isotonic with sea water (*c.* Δ1·98° C.). The other terrestrial members of the isopod group show much lower osmotic pressures (determined by the author by the same method):

	Mean Δ° C.	Range Δ° C.
Oniscus sp.	1·04 ($n=6$)	0·81–1·25
Armadillidium sp.	1·18 ($n=3$)	1·16–1·19
Porcellio sp.	1·30 ($n=3$)	1·25–1·37

The fresh-water genus *Asellus* has a blood salt concentration as low as that of other fresh-water invertebrates, with a mean freezing-point depression of Δ0·50° C. ($n=5$), range 0·41–0·62° C.

Moulting

The osmotic pressure of the blood of moulting individuals was determined in order to discover if it varied during the process of moulting. Three periods of moulting were defined: 'pre-moult', when calcium can be seen deposited in the anterior part of the integument, prior to the moult of the posterior half; 'mid-moult' between the moulting of the anterior and posterior halves; and 'post-moult' after the moulting of the anterior half. In the laboratory the time taken from the moult of the posterior to the moult of the anterior part of the integument was from 1 to 2 days. Non-moulting animals can be grouped into an 'inter-moult' stage of undetermined length.

Table 1. *Osmotic pressure of the blood of moulting stages*

Stage	Range, Δ° C.	No. of observations	Mean, Δ° C. ± S.E.
Intermoult	1·98–2·33	24	2·15 ± 0·04
Pre-moult	1·92–2·80	32	2·34 ± 0·04
Mid-moult	2·15–2·92	5	2·42 ± 0·13
Post-moult	2·25–2·87	11	2·55 ± 0·07

There is a general tendency for the osmotic pressure of the blood to rise during the moulting period. The observations which illustrate these changes are summarized in Table 1. 't' tests have indicated a significant rise between intermoult and pre-moult stages ($t=3·46$, 99% significance) and between pre-moult and post-moult stages ($t=2·47$, 98% significance). There is no indication of the sudden uptake of water after the moult which might be expected from Robertson's observations on *Carcinus* (1937). The blood of individuals kept for 2, 3 and 4 days after the moult still showed no subsequent drop in the osmotic pressure. (Observations in Table 2.)

Drying experiments

If animals are kept out of water in atmospheres of high humidity (90–100% R.H.) there is no change in the blood concentration. But if the relative humidity is lowered, there is a fairly rapid loss of water from the animal. Dry conditions for

Table 2. *Osmotic pressure of blood of individuals during moult*

Δ° C.$_{\text{blood}}$	Days of pre-moult				Day of mid-moult	Days of post-moult			
	1	2	3	4	5	6	7	8	9
Spec. 1	2·20	2·49	2·70	2·70	2·45	2·77	2·87	—	—
Spec. 2	2·26	2·42	2·74	2·80	2·92	2·86	2·85	—	—
Spec. 3	—	2·53	2·10	2·23	2·34	2·25	2·35	2·42	—
Spec. 4	—	—	—	2·14	2·26	2·37	2·38	2·50	2·46

Table 3. *Osmotic pressure of blood of individuals during drying: observations on successive days*

On wet sand	% R.H.	96	96	61	57	52	98
	Δ° C.$_{\text{blood}}$	2·12	2·10	2·20	2·47	2·98	2·46
On dry sand	% R.H.	86	93	87	79	76	98
	Δ° C.$_{\text{blood}}$	2·20	2·17	2·09	2·63	3·48	2·12

experiments were provided by suspending sulphuric acid solutions in closed breffits. The relative humidity was read from a paper hygrometer also suspended inside the jar. In one of these experiments, shown in Table 3, an individual was desiccated until Δ$_{\text{blood}}$ was 3·48° C., without any permanent ill-effects since the blood returned to its normal range of concentration within 24 hr. in standard conditions. A second animal, inadvertently desiccated until Δ$_{\text{blood}}$ was 3·75° C. (*c.* 6% NaCl), died shortly afterwards.

Immersion experiments

Ligia oceanica is not regarded as an aquatic marine animal, and various authors have reported difficulties in maintaining it in aquatic conditions. The present experiments have shown that this appears to be due primarily to asphyxiation of the animals; those which are kept in sufficiently well-aerated conditions can survive indefinitely. A suitable substratum is effective in reducing activity and so oxygen consumption.

Table 4. *Osmotic pressure of blood of individuals immersed in water*

Δ° C.$_{\text{sea water}}$	Δ° C.$_{\text{blood}}$							
	4 hr.	1 day	2 days	3 days	4 days	5 days	6 days	7 days
1·38	2·18	2·13	2·24	2·07	2·03	2·09	1·99	Dead
0·99	2·02	2·01	1·68	Dead	—	—	—	—
0·49	2·01	1·78	1·71	—	1·40	Dead	—	—

If animals are kept without aeration in 50 or 70% sea water the osmotic pressure of the blood falls appreciably (Table 4). Under these conditions the animals seldom survive for longer than a week, usually for less, but it is unlikely that the lowering of Δ$_{\text{blood}}$ is the direct cause of death since still lower values can be supported for longer periods in aerated sea water (Table 5). In 25 % sea water without aeration the values of Δ_{blood} may be lower than any recorded in Table 5, and it is possible that in this case death is directly due to excessive dilution of the blood.

When the medium is aerated or oxygenated survival in 50–100 % sea water is indefinite, and in 25 % sea water may be as much as 17 days. The figures in Table 5 refer to animals kept at 8° C., but even at 24° C. survival is indefinite in 50–100 % sea water. Experiments in which the animals were placed on filter-paper moistened with distilled water confirm the view that in the immersion experiments without aeration the cause of death is asphyxiation and not dilution of the blood. An animal taken from standard conditions was found to have a value of $\Delta_{\text{blood}} = 2{\cdot}12°$ C.; after 20 hr. on filter-paper moistened with distilled water Δ_{blood} fell to 1·77° C. and after 48 hr. to 1·44° C.; but after return to standard conditions for 24 hr. Δ_{blood} had risen to 2·15° C. Similar changes, though in less degree, were observed when the filter-paper was moistened with 50 or 25 % sea water.

Table 5. *Osmotic pressure of blood after prolonged immersion. Medium aerated*

Days	$\Delta°$ C.$_{\text{sea water}}$	$\Delta°$ C.$_{\text{blood}}$
17	0·49	1·52, 1·59
30	0·99	1·99
30	1·38	2·09, 2·10
30	1·98	2·09, 2·10
30	2·17	2·35
47	2·47	2·46, 2·41
10	2·96	3·32
3	3·46	2·95

(Normal sea water Δ° C. = 1·98.)

IV. IONIC REGULATION

The degree of ionic regulation in *Ligia* can be deduced from analyses of the common inorganic ions of the blood. Fluid from the maxillary glands was not obtained, so any possible part played by these organs in maintaining the blood concentration could not be determined. The figures for the mean values of the common ions are given in Table 6, together with those for sea water of Δ 1·98° C.

It is seen that the levels of sodium, potassium, calcium and chloride in the blood are higher than those in sea water, whereas the levels of magnesium and sulphate are lower. The total cations in the blood amount to 714 m.equiv./l. Of this total, 20 m.equiv. may be present as 'bound' calcium, since Numanoi (1934) estimates that in *L. exotica* a quarter of the calcium is bound to proteins. Total base determinations give a lower figure, 666 m.equiv./l. Total anions are 668 m.equiv./l., using the estimate of chloride obtained by Conway's method. To these might be added about 5 m.equiv. for phosphate (a single determination gave 0·073 mg. P/ml., most of which is present as HPO_4'' ion at blood pH), about 10 m.equiv. for proteinate, and about 5 m.equiv. for bicarbonate. Thus to balance 694 m.equiv. cations (714–720) there are at least an estimated 688 m.equiv. anions.

Table 6. *Concentrations of common inorganic ions in blood*

Ion	Blood			Sea water†		Concentration in blood as % of that in sea wate
	mg./ml. ± S.E.	N*	m.equiv./l.	mg./ml.	m.equiv./l.	
Sodium	13·47 ± 0·36	9	586	11·42	497	118
Potassium	0·54 ± 0·09	9	14	0·414	11	130
Calcium	1·45 ± 0·04	24	72	0·437	22	332
Magnesium	0·51 ± 0·04	14	42	1·378	113	37
Chloride‡	21·14 ± 0·66	14	596	20·58	581	103
Chloride§	23·36 ± 1·70	14	659	—	—	113
Sulphate	0·41 ± 0·01	13	9	2·874	60	14
Total anions	—	—	668	—	641	—
Total cations	—	—	714	—	643	—
Total base	—	3	666	—	643	—

* N is number of determinations.
† Δ1·98° C.
‡ Sendroy's method.
§ Conway's method.

Total concentration of ions from the data of Table 6 is 1321 mg.-ions/l. or 1385 mg.-ions/kg. water, equivalent to about Δ2·28° C. (using data on sea water from Sverdrup, Johnson & Fleming (1942, p. 67): Δ1·872° = 1·1368 g.-ions/kg. water). But direct freezing-point data for *Ligia* blood (Table 1) give a mean value of Δ2·15° C., equivalent to 1306 mg.-ions/kg. water. This difference in concentration may have resulted from different living conditions of different sets of animals, although all efforts were made to keep conditions as constant as possible. Or it may have arisen from a seasonal difference in the concentration of the blood, since Widmann (1935) gives a range, for males, from Δ2·20° C. in August to 2·33° C. in November, and says that there is a fall in the concentration in April. Of the data given here, the osmotic pressure measurements were all made in April, May and June, while the inorganic analyses were made in October, November and December.

From the water content of *Ligia* blood the total solids can be calculated as 7·35 %, of which salts (from analysis) account for 4·04 %. A rough approximation of the protein content would thus be 3·3 % of the weight of the blood (33 mg./g.).

The relative proportions of cations bears some relationship to the physiological regulation of the animal (Florkin, 1949, p. 98) and perhaps to its activity (Robertson, 1949). The value of the ratio Na + K/Ca + Mg in the blood of many marine invertebrates does not differ appreciably from that in sea water, 3·8. For example, in the stone crab *Lithodes*, the blood has a value of 3·8, and it is very similar in composition to the sea water in which it lives; but other decapod crustaceans may show very different values, such as *Palinurus*, 9·2. *Ligia* occupies an intermediate position with a value of 5·3.

V. GENERAL DISCUSSION

Ligia oceanica is able to regulate the osmotic pressure of the blood when completely immersed in 75 or 100 % sea water provided that sufficient oxygen for the regulatory process is available. The blood is kept fairly constant in composition over this range, but in lower or higher concentrations Δ_{blood} follows that of the outside medium· In terrestrial conditions the humidity of the environment is important in determining the blood concentration; water appears to be lost readily when the relative humidity of the air drops below 90%, if the animal has no access to water; there seems to be little control of water loss in dry air. Any water which is available is absorbed, and if only distilled water is present, the blood concentration is reduced. The general osmotic behaviour of *Ligia* does not lead us to suppose that it has any special physiological mechanisms which might enable it to embark successfully on a truly terrestrial existence. It is rather a marine animal which has developed a high degree of tolerance to internal osmotic changes, whether they are brought about by drying or by external osmotic changes. The evaporation of water in hot terrestrial conditions may, as Edney (1952) suggests, provide an adaptation for the cooling of the animal. It cannot be concluded from the evidence above that this species is intermediate either between the marine and the terrestrial members of the group or between the marine and the fresh-water members.

In the domain of ionic regulation it appears that *Ligia* can excrete or exclude magnesium and sulphate efficiently; calcium is accumulated to a notable extent, but about 25% of the total calcium may be bound to proteins; sodium, chloride and potassium are all higher in the blood than in sea water, especially potassium. The relative proportions of the inorganic cations indicate a highly developed inorganic regulation, comparable with that of some of the decapods.

Whether the urine is isotonic with the blood, as in marine and brackish-water crustaceans, or hypotonic to the blood, as in most fresh-water crustaceans, the problem of the source of salts remains. There are two possibilities: (1) that salt is absorbed from ingested food, and (2) that it is absorbed from the environment by some special region of the body. The first suggestion is a probable one, since the animal usually feeds on seaweed detritus, with a high salt content. But *Ligia* is reported 450 ft. above sea-level in the Outer Hebrides (Fraser Darling, 1947, p. 215); although seaweed detritus may be absent here, it presumably feeds on salt-encrusted vegetation. Absorption from the environment is possible either by swallowing the medium and absorbing it from the gut, or by selective absorption on some part of the integument. *Ligia* has a curious habit of dipping the uropods into water and draining the fluid over the pleopods. This behaviour may be merely a device for keeping the respiratory surfaces wet, but may also serve to regulate salt.

VI. SUMMARY

1. Osmotic pressure of the blood of *Ligia oceanica*, measured by the freezing-point depression, has a mean value of $\Delta 2{\cdot}15 \pm 0{\cdot}04°$ C. ($\equiv 3{\cdot}58\%$ NaCl on weight/volume basis).

2. Osmotic pressure of *Ligia* blood is much higher than that of other terrestrial isopods: *Oniscus* sp. $\Delta 1{\cdot}04°$ C.; *Armadillidium* sp. $\Delta 1{\cdot}18°$ C.; *Porcellio* sp. $\Delta 1{\cdot}30°$ C. or of the fresh-water *Asellus* sp. $\Delta 0{\cdot}50°$ C.

3. The osmotic pressure of the blood increases during the process of moulting, but no subsequent decrease is observed in the 4 days following.

4. Animals kept at low humidities lose water. They may be desiccated without permanent adverse effects until Δ_{blood} is 3·48° C. (≡5·8% NaCl). Recovery to a normal level takes about 24 hr. in moist conditions.

5. In well-aerated sea water between 50 and 100% concentration, animals survive without much alteration in Δ_{blood}. Above and below this range Δ_{blood} rises and falls.

6. In animals kept on filter-paper moistened with distilled water Δ_{blood} may fall to 1·44° C. (≡2·4% NaCl) without permanent adverse effects.

7. Analyses of inorganic ions in the blood show that sodium, potassium and chloride are all higher in concentration than in sea water; calcium is much more concentrated; and magnesium and sulphate much reduced.

I should like to acknowledge the assistance of Dr J. D. Robertson, Dr J. A. Ramsay, and Prof. H. Munro Fox. During the course of part of this work I was in receipt of a Science Scholarship from the University of New Zealand.

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THE FORCES EXERTED BY THE TUBE FEET OF THE STARFISH DURING LOCOMOTION

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(*Received* 21 *April* 1953)

I. INTRODUCTION

The nineteenth-century view (Lamarck, 1840; Van der Hoeven, 1856; Dujardin & Hopé, 1862; MacBride, 1906) concerning the locomotion of the starfish was that the animal extended a tube foot forwards, attached the disk to the ground and then contracted the foot, thus pulling the animal along. This will be called the 'traction theory'. Jennings (1907) did not agree with this view since he found that a starfish could walk as well over sandy or greasy surfaces as it could over rocks. He suggested that the tube foot acted as a lever and propelled the animal forwards. This will be called the 'lever theory'. Later accounts, such as that given by Flattely & Walton (1922), although mentioning Jennings's view give more credence to the traction theory.

There is no doubt that the tube feet can attach themselves very firmly to the substratum. Paine (1926) showed that the force required to tear a tube foot from its attachment had an average value of 29 g., and Smith (1947), investigating the mechanism of attachment during the step, showed that there was a special series of nerves and muscles that took part in disk attachment. Both these writers assumed that it was normal for the foot to attach firmly to the substratum during locomotion, yet, if Jennings is correct, the tube foot need obtain no more than a slight purchase on the ground by means of its disk.

The purpose of the present investigation was to demonstrate the truth or otherwise of these theories by recording the forces exerted by a single tube foot during its period of contact with the ground.

II. METHODS

(1) *Direct observation and cinematography*

The common starfish, *Asterias rubens* L., was watched moving over rocky and sandy surfaces on the beach and also climbing in glass tanks in the aquarium. To aid further analysis of the walking movements cine films were taken both from the side and from below. A Sinclair 35 mm. camera was used at speeds of 8–24 frames a sec. and illumination was provided by two Photoflood lamps. In some cases increase of contrast was provided by staining the animal with Nile Blue sulphate, this stain having previously been shown to have no harmful effects on the animal. The starfish usually showed a definite response when the Photoflood lamps were switched on, but after some time it became adapted to the light and moved about in a normal manner. In all cases the results obtained from the stained animals and from cine films were confirmed on normal walking animals since some authors (Manton, 1951) have shown that animals may react abnormally under intense illumination.

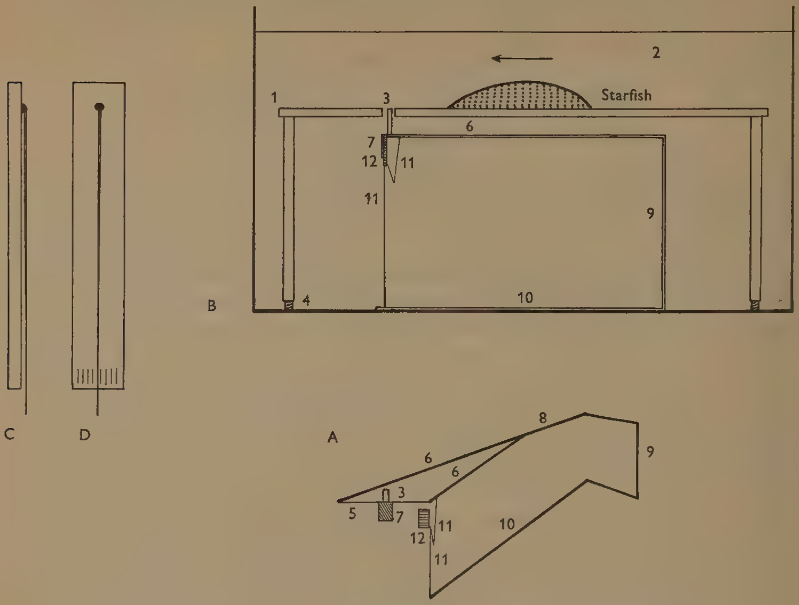


Fig. 1. Apparatus for measuring the forces exerted by a single tube foot. For description see the text.

(2) *Measurement of the forces*

The forces exerted by a single tube foot were measured with the animal (*a*) walking upon a horizontal surface, and (*b*) climbing upon a vertical surface. It is therefore necessary to define the terms which will be used to describe the direction of the measured forces.

Longitudinal forces are measured in the direction of progression; if the force is such as to assist progress then it is said to be *positive*: if it retards progression it is called *negative*.

Forces acting at right angles to the surface of the ground are called *normal forces* and are measured as pull or push, i.e. if an animal is walking upon a horizontal surface its weight pushes on the ground.

The apparatus is illustrated in Fig. 1 A, B. A perspex platform (1) is set up in a tank of sea water (2). A small hole, about 5 mm. in diameter, is bored through the Perspex, and a short glass rod 2 mm. in diameter is passed through the hole. The height of the platform can be adjusted by screws (4) so that the top of the rod is flush with the upper surface. The rod is mounted on a thin glass fibre (5) which is held under tension between glass rods (6). A mirror (7) is also mounted on this fibre. This system forms a simple torsion balance which responds to the longitudinal forces. The response to the normal forces is provided for by the compliance of the framework of glass rods (6), (8), (9) and (10). The free end of rod (10) is connected to one of the rods (6) by a bent glass fibre (11) which carries a second mirror (12). Fibre (11) is drawn so as to be thinnest at the point at which the mirror is attached. Movement of rod (6) in relation to rod (10) bends the fibre (11) and deflects the mirror. A beam of light coming from a galvanometer lamp falls on the mirrors and is reflected to a revolving drum covered with photographic paper, at 3 m. distance. Even at this distance the maximum deflexion recorded was 4 cm. (the usual deflexion was 1 cm.) and the apparatus may therefore be said to be isometric. Interruption of the light beam by a metronome provides the time scale.

For purposes of calibration, which must be carried out under water, an additional piece of apparatus is required (Fig. 1 C, D). This is a glass fibre attached to a piece of Perspex provided with a scale. This apparatus is first calibrated in air by the attachment of weights to the fibre and the relation between the deflexion and the weight is established. The end of the fibre is then pressed against the rod (3) in appropriate directions and the deflexion of the light beam is recorded against the deflexion of the fibre.

Several different models of this apparatus were built with various minor modifications. In the model which was used for most of the experiments the sensitivity (in terms of the smallest deflexion that could be measured on the record) was 2·5 mg. for the longitudinal forces and 25 mg. for the normal forces. For the more accurate measurement of the normal forces discussed on p. 582 the sensitivity of the model used was 5 mg.

The animal was allowed to walk upon the horizontal Perspex platform as shown in Fig. 1. It was possible to turn the whole apparatus through 90°, and in this case the animal had to climb up a vertical surface. In order to facilitate direct observation of events the animal was initially placed in such a position that one of the lateral arms rather than the leading arm would traverse the hole in the Perspex. When one of the tube feet was seen extending towards the rod (3) the drum was started. If the foot made contact with the rod it was carefully watched throughout the whole period of contact. Occasionally two tube feet came into contact with the rod at the same time and when this happened the record was discarded.

The usual type of record obtained from an animal walking over a horizontal surface is shown in Fig. 2A. The upper trace is of the normal force whilst the lower trace is of the longitudinal force. Fig. 2B shows the same record after analysis with the force plotted against time.

III. RESULTS

(1) *Direct observations and cine films*

In 1947 Smith described the sequence of events that occurred during the stepping of the tube foot without committing himself to either the traction or the lever theory. The tube foot system consists of two units, the tube foot externally and the ampulla internally. The tube foot goes through the following phases during the step.

(1) *Pointing or orientation.* The tube foot is pointed in the direction of locomotion.

(2) *Protraction.* The tube foot is extended in the direction of locomotion by contraction of the ampulla.

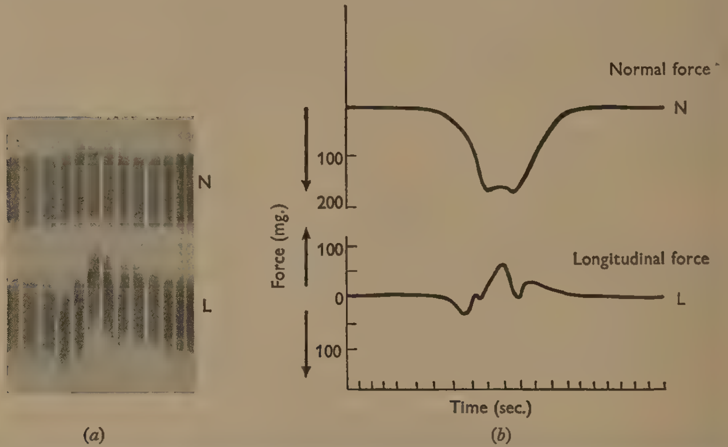
(3) *Attachment.* The tube foot comes into contact with the ground and the disk attaches.

(4) *Swing.* This is the active phase of the step in which the disk remains fixed in position whilst the base of the foot moves over the point of attachment.

(5) *Detachment.* The disk is removed from the ground.

(6) *Retraction.* The tube foot retracts by contraction of the longitudinal retractor muscles in the tube.

(7) *Repointing.* The tube foot points once more in the direction of locomotion.



(*a*) (*b*)

Fig. 2. Record of the forces exerted by the tube foot of an animal walking upon a smooth horizontal surface. (*a*) The record obtained from the apparatus. The upper trace is of the normal force, the lower trace is of the longitudinal force. The time interval is in seconds and the record reads from left to right. (*b*) The same record plotted as force against time.

A push downwards is registered as an upward excursion of the trace. In (*b*) the normal force has been inverted so that push downwards is plotted downwards. The convention, that push is plotted downwards and pull is plotted upwards, is observed in subsequent figures.

Direct observations of the behaviour of the tube feet in an animal walking upon a horizontal surface agree with the account given by Smith. Smith never saw what he called the 'ideal step' in which all the phases occurred one after the other; instead retraction and repointing always occurred simultaneously. Analysis of the cine films shows that though this is true for the majority of cases, in a minority of cases one finds the retraction occurring before the repointing, i.e. the 'ideal step' does occur. From the films it is possible to trace the positions of the tube foot through the different phases of the step cycle. These records confirm the evidence of direct observation and provide some additional information (Fig. 3). Ideally the base of the foot should swing directly over the point of fixation of the disk. This movement is rarely seen in the photographs; instead it is more usual for the base to pass somewhat to the side of the disk. The tube feet often bend all along their length and not only at the base as Smith describes. The bending of the tube feet during the attached phase is suggestive evidence against the traction theory. Furthermore, the body of the animal is raised off the ground when it walks upon a horizontal surface and this would not be possible unless the feet act as struts.

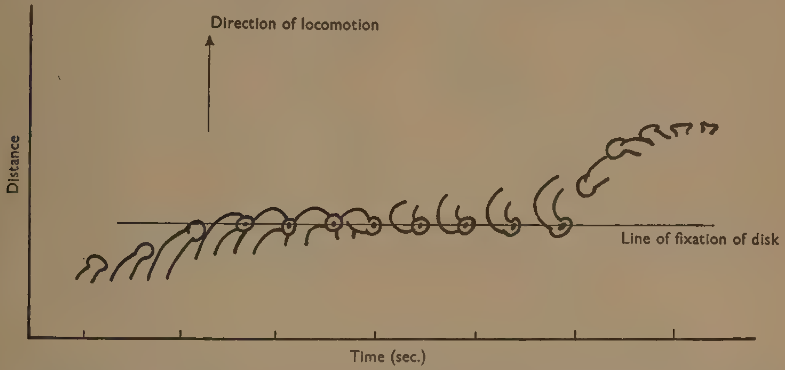


Fig. 3. The successive positions of a tube foot during the step, as traced from cine films. The foot is protracted and attached to the ground, the disk then remains fixed in position whilst the base and the body are moved over it. The foot is then released from the ground, retracted and repointed. Note that the foot becomes bent during the attached period of the step. The time interval between each of these positions is 0·5 sec.

(2) *Measurement of the forces exerted by a tube foot*

(*a*) *Locomotion in a horizontal plane*

Fig. 4*a* shows the forces exerted by a single tube foot during the step cycle. The upper graph shows the normal whilst the lower graph shows the longitudinal forces. The normal forces show that throughout the attached part of the step the tube foot pushes downwards. The time course of the longitudinal force is diphasic, indicating an initial retardation (negative) followed by an acceleration (positive). Similar records are obtained when the animal walks over a sandy surface (Fig. 4*b*). In all these cases the normal force is exerted as a push downwards and the record of the longitudinal force is diphasic. This diphasic record is similar to that obtained by Fenn (1930) from the human foot. In both cases there is an initial retardation phase followed by an acceleration phase.

Records of this type are incompatible with the traction theory. They show that the tube feet certainly act as struts and may also act as levers.

In about 20% of the records events follow a different course; at the end of the step the normal force changes from push to pull, and in the case of the longitudinal force the usual positive phase is replaced by a second negative excursion (Fig. 4*c*). This is most probably due to the disk becoming firmly attached to the rod and then failing to become detached when the foot starts to retract and repoint. Another uncommon type of record is seen in Fig. 4*d*. The longitudinal force shows only the positive phase whilst the normal force shows that the foot exerts a steady pull upwards, i.e. it does not support the weight of the body. This step, like the others on the horizontal plane, has an attached phase lasting 7–10 sec. It occurs in about 3 % of the traces and except in its short duration it resembles some of the records obtained with the animal on a vertical surface, as described below.

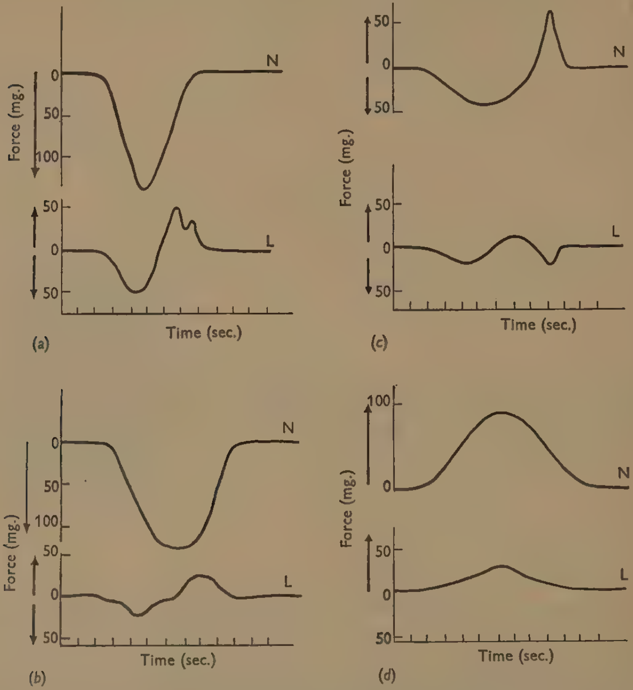


Fig. 4. Record of the forces exerted by the tube foot of an animal walking upon a horizontal surface. For description see the text.

(*b*) *Locomotion in a vertical plane*

When a starfish walks up a vertical surface the records obtained are entirely different (Fig. 5). In the first place the step is very much slower and has an attached phase of 20 sec. compared with that of 7–10 sec. for the step on a horizontal plane. In the second place the foot exerts a normal force pulling the animal towards the substratum. In the third place the diphasic pattern is no longer seen in the longitudinal force; there is no negative phase, but instead the foot develops a positive force throughout its period of attachment. Direct observation of such animals show that the tube foot usually becomes detached from the ground as soon as the disk passes directly below the base of the foot.

Records of this type are incompatible with the lever theory. They show that the tube foot does not act as a strut; instead the data are best explained in terms of the traction theory.

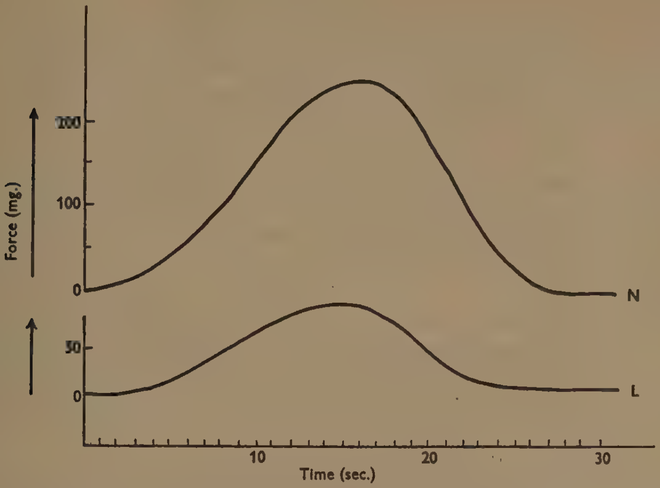


Fig. 5. Record of the forces exerted by the tube foot of an animal walking up a vertical surface. For description see the text.

Thus when the animal walks up a vertical surface the tube foot does not act as a strut or lever but instead protracts, attaches to the ground and then contracts, so pulling the animal forwards. A similar result can be obtained by allowing the starfish to walk over a horizontal surface opposed by a force equivalent to that of its own body weight in water.

IV. DISCUSSION

As mentioned in the Introduction there are two theories concerning the mode of action of the tube feet. According to the traction theory the foot is extended, attached and then contracted—so pulling the animal along. According to the lever theory the tube foot acts as a strut upon which the animal stands, and this strut can be used as a lever to propel the animal along in much the same way that a man uses his leg.

The results show that both modes of action can be used by the starfish. When the animal is on a horizontal surface it raises its body off the ground on the tube feet. The feet thus act as a series of struts. The results, however, do not definitely show that the feet act as levers. In theory it would be possible to demonstrate the lever action in the following way. If the rate of progress is uniform the records of the longitudinal forces, in which force is plotted against time, can be treated as records in which force is plotted against distance. If the area under the first (retardation) phase of the graphs is subtracted from the area under the second (acceleration) phase of the graph there should remain a small positive quantity which represents the work done by the tube foot in assisting the animal's progress. This type of analysis was, in fact, carried out for a number of records and it was found that the work done in retardation slightly exceeded the work done in acceleration but the difference was not statistically significant. When one examines the problem more closely it becomes obvious that a clear demonstration of the lever action is unlikely to be given by an investigation of this type. A rough calculation shows that the force required to drag a starfish through the water at its normal rate of progress (1 mm./sec.) is less than 0·2 dyne, so that even if all this force was exerted by the single tube foot under observation it would be too small to be registered by the most sensitive apparatus used in these experiments. If one tries to obtain a measurable force by increasing the resistance to progress, the animal changes over to the traction method.

When the tube feet act as a series of struts they must support the body weight. If one knows the average weight supported by a tube foot throughout the attached phase of the step cycles and the weight of the animal in water, one can calculate the number of feet supporting the body. Analysis of one hundred steps shows that the average value of the downwards thrust during the attached phase is 0·04 g. Then in an animal weighing 3·25 g., the body weight is supported by 81 feet (i.e. 3·25/0·04) though the animal has a total complement of well over a thousand feet. Since the attached phase of the step only occupies one-third of the step cycle, it means that at most 300 tube feet take part in locomotion when the animal is moving quickly (2 mm./sec.). The other tube feet are either resting, protracting and searching for the ground, or pointing towards the mouth. A rough estimate of the number of tube feet in contact with the ground can be obtained from photographs. In the case of quickly moving animals figures ranging from 60 to 120 were obtained, which is in reasonable agreement with the figure of 81 arrived at above. When the animal walks more slowly each tube foot carries less of the body weight and there are more tube feet in contact with the ground. This decrease in the number of tube feet supporting the quickly moving animal agrees with the results obtained in Arthropod locomotion.

V. SUMMARY

1. Doubt has existed as to whether the tube feet of a starfish in locomotion act as a system of levers or whether they form a traction system, attaching to the substratum and contracting so as to pull the animal along.

2. This problem has been investigated by recording the forces exerted by a single tube foot. The recording apparatus is described.

3. It is shown that both lever and traction methods can be used according to circumstances.

(*a*) When the starfish is walking on a horizontal surface it is shown that the tube feet certainly act as struts and that they probably also act as levers.

(*b*) When the starfish is climbing upon a vertical surface (or meeting with resistance upon a horizontal surface) the tube feet attach themselves to the substratum and form a traction system.

It is a pleasure for me to acknowledge the help and encouragement given by Dr J. A. Ramsay throughout this work. I am also very grateful to Dr F. S. J. Hollick and Dr G. D. S. MacLellan for their advice during the construction of the apparatus. The work was done whilst holding a Research Fellowship at Pembroke College, Cambridge.

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LABORATORY EXPERIMENTS ON GREGARIOUSNESS DURING SETTING IN *BALANUS BALANOIDES* AND OTHER BARNACLES

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(*Received* 27 *March* 1953)

(With Plate 16)

1. INTRODUCTION

Cirripede larvae must become widely dispersed during their long planktonic life, yet the adults of many species are found exclusively in restricted habitats. Some are epizoic and others are parasitic on specific hosts, which the cypris larvae must therefore seek out and recognize, whilst cyprids of many Lepadidae and Rhizocephala are known to seek out adults of their own species, to which they attach themselves as dwarf or complemental males. Burton (1949) pointed out that cyprids of *Balanus balanoides* (L.) seem to be gregarious during setting, and field experiments indicated similar behaviour in *Elminius modestus* Darwin (Knight-Jones & Stevenson, 1950). The following experiments have established that these common littoral forms are able to recognize their own species, and have thrown some light on the mechanism of recognition.

2. METHODS

Cyprids of *Balanus balanoides* were collected near Bangor during April and May, by picking up *Fucus* from shallow water and rinsing the fronds in wide-mouthed jars (breffits), which contained about 2 l. of sea water. Large numbers of cyprids were dislodged from temporary attachment to the fronds, and were later pipetted away to clean vessels, taking advantage of their positive phototaxis.

Cyprids of *Balanus crenatus* Bruguière and *Elminius modestus* were obtained from plankton hauls. They could be readily separated from the rest of the catch, as they became trapped in the surface film when left undisturbed in shallow dishes. Apparently the cyprid cuticle is more strongly hydrophobic in these species than in *Balanus balanoides*.

Experiments were carried out in uniformly illuminated glass dishes, containing 250 ml. of sea water, advantage being taken of the fact that glass is unfavourably smooth for the setting of barnacles, especially of *B. balanoides* (Runnström, 1925; Barnes, Crisp & Powell, 1951). Records were made of the numbers setting on surfaces which had been treated in different ways. Cyprids were usually active, alternately swimming and crawling. Their exploratory habits enabled them to choose between two surfaces presented to them in the same dish. A cyprid which was about to settle could be recognized because it crawled slowly and turned frequently (Doochin, 1951), as do the larvae of *Ostrea* (Cole & Knight-Jones, 1939) and *Spirorbis* (Knight-Jones, 1951), when they are about to settle. After setting, no further locomotion was possible, and a settled cyprid could easily be recognized by its firm attachment and obtuse anterior end. The interval between setting and the moult which accompanied metamorphosis was about 24 hr. in *Balanus balanoides*, somewhat less in *B. crenatus*, and less than 4 hr. in *Elminius modestus*.

3. DELAYED SETTING IN ABSENCE OF OWN SPECIES

It was repeatedly shown that cyprids did not settle readily except on surfaces which already bore either barnacles of their own species or the persistent bases left after these barnacles had been removed. The following experiment proved that failure to settle was not due to development being insufficiently advanced, and that setting could be delayed for long periods without ill effects. A hundred and forty freshly collected cyprids of *Balanus balanoides* were divided equally between seven dishes, and a *Mytilus* valve bearing the bases of freshly detached barnacles was placed in one of the dishes on the following day. Similar valves were added, at intervals of 4 days thereafter, to each of the other dishes in turn, and notes were made every day of the condition of the cyprids and of the numbers settled on the valves (Table 1). None settled on the glass of the dishes.

In the first dish to receive a *Mytilus* valve the majority of the larvae settled within 24 hr., and it is therefore probable that the majority of those in other dishes would have settled within this period if a suitable substratum had been available. Where none was available the planktonic life was prolonged by nearly 2 weeks without serious ill effects, for most cyprids presented with a valve after this period were still able to settle within 24 hr. and to metamorphose. These metamorphosed individuals, though somewhat below the normal size, seemed healthy and started to grow, but after further prolongation of the planktonic life the majority of cyprids became sluggish, encumbered by bacterial growths, and apparently unable to settle.

4. SPECIFIC NATURE OF GREGARIOUS RESPONSE

Seven parallel series of ten experiments were set up, each experiment with a similar batch of twelve *B. balanoides* cyprids and one *Mytilus* valve, from which the flesh had been removed. In three series of experiments the *Mytilus* valves bore live individuals of *B. balanoides*, *B. crenatus* or *Elminius modestus* respectively. In another three series they bore bases only of these species, and in the last series they bore no trace of barnacles (Table 2A). The valves were all similar and had been collected in such a way as to ensure that the absence of *Balanus balanoides*, from those which were bare or which bore other species, was due to geographical distribution, rather than to the valves being intrinsically less suitable for setting. Thus some had been collected very early in the season from Cardigan Bay, and these bore numerous newly settled *B. balanoides*; whilst the others, which had been collected at about the same time from Bangor, Conway and Rhyl, bore no *B. balanoides* because it was too early in the year for setting of this species to the east of Anglesey, where liberation of larvae regularly occurs later in the season (Crisp, 1953). The experiments showed that the other species of barnacles were comparatively ineffective in promoting setting, though they appeared to be better than nothing. The fact that more cyprids settled on valves bearing *B. crenatus*, than on those bearing *Elminius modestus*, may possibly have been associated in some way with the closer relationship of the former to *Balanus balanoides*. Alternatively, it may have been due to the cyprids reacting to surface irregularities, since the individuals of *B. crenatus* were larger and more numerous than those of *Elminius modestus*, whilst their calcareous bases remained on the valves after the barnacles had been removed, making the surfaces rougher.

Table 1. *Numbers of* Balanus balanoides *settled on successive days, out of similar batches of twenty cyprids collected at the same time and kept in separate dishes A, B, C, D, E, F and G. A* Mytilus *valve which bore bases of barnacles was added to each dish on the date marked with an asterisk*

Date	A	B	C	D	E	F	G
April 26	Cyprids collected from shore						
27	0*						
28	15						
29	16						
30	18						
May 1	19	0*		All swam occasionally			
2	20	17					
3		19					
4		19					
5		19	0*	All swam when disturbed			
6		19	15				
7		19	18				
8		20	18				
9			18	0*	Most swam when disturbed		
10			18	15			
11			18	17			
12			18	17			
13			18	17	0*	Many swam weakly	
14			18	17	2	when disturbed	
15			18	17	4		
16			18	17	5		
17			18	17	5	0*	All capable
18			19	17	5	1	of weak
19			19	17	6	2	movements
20			19	17	6	2	
21			19	17	6	2	0*
22			19	17	6	2	0
26			19	17	6	2	0
Totals metamorphosed	20	20	19	17	6	2	0

Similar experiments, with similar results, were carried out with pieces of sandstone from Brixham, Devon, which bore either *Balanus balanoides*, *B. perforatus* Bruguière, *Chthamalus stellatus* (Poli), or *Verruca stroemia* (O. F. Muller) (Table 2 B). Fewer cyprids settled in these experiments because the adult *Balanus balanoides* were few, whilst the stones which bore them were so crowded with *Chthamalus* as to leave little space for setting.

Experiments in which cyprids of various species were offered a choice between two *Mytilus* valves placed in the same dish, one bearing bases of their own species and the other bases of another species, showed that the larvae can select surfaces bearing their own species, in preference to those bearing another (Table 3). The experiments with *Elminius* cyprids, which were carried out at Burnham-on-Crouch, involved choice between *Mytilus* valves from Rhyl, which bore *Elminius* and a few *Balanus*, and similar valves from Bangor, which bore *Balanus* only, because *Elminius* had not spread as far as Bangor. Some of the valves had been stored in 5% formaldehyde for 4 days, between the time of collection and the time of the experiments, and it is interesting to note that the specific effect was unaffected by this treatment (cf. p. 594).

Table 2. *Numbers of* Balanus balanoides, *out of similar batches of twelve cyprids, setting within 24 hr. in:*

(A) Seven parallel series of ten experiments with *Mytilus* valves bearing:

One alive and several recently separated bases of			None alive but several recently separated bases of			No trace of barnacles
B. balanoides	*B. crenatus*	*E. modestus*	*B. balanoides*	*B. crenatus*	*E. modestus*	
9	5	0	1	0	0	0
12	5	0	9	0	0	0
9	0	7	5	0	0	0
6	0	0	4	0	0	0
9	5	1	7	4	3	1
12	1	0	12	0	0	0
9	8	0	10	4	0	0
12	5	6	8	4	3	0
12	7	0	12	0	0	3
5	1	2	2	2	0	0
95	37	16	70	14	6	4

(B) Four parallel series of eight experiments with similar pieces of sandstone rock bearing:

B. balanoides and many *Chthamalus*	*B. perforatus* only	*Chthamalus stellatus* only	*Verruca stroemia* only
4	1	0	0
6	0	0	0
0	0	0	0
4	0	0	0
0	1	0	0
4	0	1	0
3	0	0	0
1	0	0	0
22	2	1	0

The positive results of these experiments on choice indicated that reception of the stimulus to settle requires close proximity to, or contact with, the previously settled individuals or their bases. They further suggested that cyprids which have received this stimulus rarely swim away to another surface before setting. Subsequent experiments (p. 591) confirmed that contact was necessary for the reception of the stimulus, and repeated observations showed that cyprids which came into contact with barnacles or bases embarked immediately upon the turning movements which characterize the setting action pattern. Their subsequent migrations were usually limited, as is illustrated by the experiments described in the next section.

Table 3. *Numbers of cyprids setting in four series of eight experiments, which involved choice between* Mytilus *valves bearing bases of their own species and similar valves bearing bases of another species.*

Cyprids of *B. balanoides* with bases of		Cyprids of *B. crenatus* with bases of		Cyprids of *E. modestus* with bases of		Cyprids of *E. modestus* with formalin-preserved bases of	
B. bal.	*B. cren.*	*B. cren*	*B. bal.*	*E. mod.*	*B. bal.*	*E. mod.*	*B. bal.*
6	0	3	3	15	0	9	4
12	0	6	0	8	1	5	0
9	0	3	1	9	1	2	0
12	0	3	1	5	1	1	0
8	0	4	2	12	2	3	0
4	0	5	0	8	3	3	0
4	1	2	0	4	0	4	1
12	0	5	0	5	0	2	0
67	1	31	7	66	8	29	5

5. LOCALIZATION OF GREGARIOUS RESPONSE

A slate which bore a small patch of adult *Balanus balanoides* (Pl. 16, fig. 1) was taken from a shore before heavy settlement of barnacles had begun there. The position of the patch was outlined by a scratched groove, and the barnacles were removed, leaving only the almost invisible bases. The slate was left overnight, exposed to cyprids in a large vessel, lying horizontally and illuminated from above. The cyprids, which started crawling over the whole surface of the slate, settled almost exclusively within the area outlined (Pl. 16, fig. 2) which suggests that little or no migration occurred after contact with the bases. A similar experiment was carried out with a slate which bore *B. crenatus* as well as *B. balanoides* to illustrate the ability of the cyprids to distinguish their own species (Pl. 16, figs. 3, 4). In this experiment the positions of the barnacles were not outlined before removal, because they were rather scattered, and because the calcareous bases of *B. crenatus* were fairly conspicuous, whilst the relative positions of the other bases were known from the photograph of the intact barnacles. Comparison of figs. 3 and 4 (Pl. 16) shows that few cyprids settled in the areas which had been occupied by *B. crenatus*, many in those which had been occupied by *B. balanoides*, and many in grooves and depressions nearby, to which they had presumably crawled.

It might be expected that cyprids encountering barnacles on surfaces which are otherwise unfavourable for setting would receive the stimulus to settle, but would swim off during their final reconnaissances, and become pioneer colonizers of neighbouring bare surfaces. An opportunity to demonstrate this occurred when two dishes, in which experiments had been carried out, were each found to contain a recently metamorphosed *B. balanoides* attached to the glass. Very few cyprids of this species (much less than 0·1 %) settled on bare glass in the whole course of this work, but once these abnormally undiscriminating individuals had begun colonization of the glass surfaces others settled beside them when successive batches of numerous cyprids were introduced into the two dishes. After about twenty had settled in each dish, the majority of the cyprids which were introduced attached themselves to the adjacent glass within 2 or 3 days, there being no other surface available. When each dish bore about fifty settled barnacles, the two dishes were matched with two other dishes which bore none. Similar batches of cyprids were added to all four dishes, and similar stones of rough granite were suspended freely in the water by cotton threads. Cyprids which had encountered the barnacles in the experimental dishes could reach the stones only by swimming, yet setting occurred much more readily on these stones than on those in the other dishes (Table 4). Contact with the settled barnacles appears to have provided a sensitizing stimulus, so that subsequent contact with the rough stones induced the final response. Setting of barnacles normally involves responses to multiple stimuli, being delayed by crowding and orientated by light and currents (Crisp, 1953), and the extent to which setting is localized, after contact with adults, evidently depends upon the presence of a favourable surrounding surface.

Table 4. *Numbers of* Balanus balanoides *setting within 24 hr. in two series of fourteen experiments, each with similar batches of twelve cyprids presented with granite chippings, which were suspended in dishes containing: (a) no settled barnacles; (b) about fifty recently settled barnacles on the glass walls*

								Totals
(*a*)	0	0	3	0	0	0	0	3
	0	0	0	0	0	0	0	
(*b*)	3	1	7	0	7	1	2	35
	0	0	2	7	2	1	2	

6. RESPONSE TO FRAGMENTS OF BARNACLES

To see if any part of the body other than the basis would induce setting, pieces of adult barnacles were placed on top of similar freshly quarried granite stones, which were offered to cyprids of *B. balanoides* in four parallel series, each of twenty experiments. In a parallel control series (*a*) there were no fragments on the stones. In the four experimental series respectively, each stone bore (*b*) two detached adults of *B. crenatus*; (*c*) two detached adults of *B. balanoides*, complete except for their bases; (*d*) the bases of about twenty detached adults, scraped with a scalpel off smooth pebbles or shells, and scattered on the granite in small, flocculent heaps; (*e*) the bodies of three adults extracted from their shells, including the cirri and viscera (Table 5A). No cyprids settled on the untreated stones, but significant numbers settled on those which bore fragments of their own species. A few cyprids settled beside the adults of *B. crenatus*, giving further slight evidence that setting occurs more readily in the presence of barnacles of other species, than in the complete absence of previously settled barnacles (cf. p. 586).

Table 5. *Numbers of* Balanus balanoides, *out of similar batches of twelve cyprids, setting within* 24 *hr. in:*

(A) Five parallel series of twenty experiments with rough granite stones which were:

											Totals
(*a*) Untreated	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	
(*b*) With detached *B. crenatus*	0	1	0	0	0	0	0	0	1	0	4
	0	1	0	1	0	0	0	0	0	0	
(*c*) With detached *B. balanoides*	5	2	1	0	0	0	0	2	0	0	17
	0	2	1	1	0	1	0	2	0	0	
(*d*) With bases scraped off shells or pebbles	3	0	0	6	0	0	1	0	5	2	19
	0	0	0	1	0	1	0	0	0	0	
(*e*) With bodies extracted from their shells	0	1	0	1	2	0	0	3	0	0	9
	0	0	1	0	1	0	0	0	0	0	

(B) Two parallel series of experiments with pieces of slate which were:

(*a*) Untreated	0	0	0	0	0	0	0	0	0	0
(*b*) Smeared with squashed cyprids of *B. balanoides*	1	4	0	0	3	1	0	3	0	29
	2	3	0	0	2	3	4	2	1	

The cyprids setting alongside the adult cirri and viscera, with which they do not normally come into contact, indicated that setting occurs in response to some substance which is generally distributed throughout the body. Since bases alone promote setting, the active substance is probably cuticular, for the attachment cement which largely constitutes the basis is similar in properties to the cuticle, and the cement glands are regarded as modified tegumental glands (Thomas, 1944). Cyprids have relatively large cement glands communicating with the antennules, and when dissected with needles they yield considerable quantities of a sticky substance. In a series of experiments slates which had never borne barnacles were broken into paired pieces, and one piece of each pair was presented without treatment to a batch of cyprids, whilst the other was presented to a similar batch after having been smeared thoroughly with from ten to twenty dissected cyprids, left for a few hours in sea water and then rinsed. Twenty-four hours later none of the cyprids had settled on the untreated slates, but in most batches several had settled on slates smeared with squashed cyprids (Table 5B). It is not certain that they were responding solely to the extruded cement, since fragments of the cyprids' bodies were inevitably mixed with this.

The numbers of cyprids setting in these experiments (Table 5A and B) were comparatively small, and this reluctance to settle could hardly have resulted from a phobic response to damaged tissues, because placing damaged barnacles amongst attached adults did not in the least discourage setting. Cyprids reacted readily only to cuticular material which was firmly attached to a surface, presumably because it was more readily encountered by their groping antennules, or perhaps because looseness of a surface discourages setting. When kept crowded for a long time they often clung to one another in temporary aggregations, but such contact did not make them more ready to settle, which suggests that they respond only when crawling.

7. NEED FOR CONTACT TO INDUCE SETTING

The following experiments were carried out to test whether the gregarious response depends on chemosensory perception of a water soluble substance emanating from settled barnacles, or whether contact with the barnacles is necessary. Small shells or stones bearing many settled barnacles were enclosed in bags of bolting silk, to prevent cyprids from touching the barnacles, and each bag was placed in a dish containing a batch of cyprids and a *Mytilus* valve. These dishes were grouped in two series of experiments, one series with *Mytilus* valves bearing bases of freshly detached barnacles, and the other with valves bearing no bases. Setting readily occurred upon the valves which bore bases, but very few cyprids settled upon the others; indeed, not significantly more than in a third series of experiments in which valves without bases were offered to cyprids in dishes without bags of settled barnacles (Table 6A). It appeared that there was nothing to encourage setting in the water which surrounded the settled barnacles.

Table 6. *Numbers of* Balanus balanoides, *out of similar batches of twelve cyprids, setting within 24 hr. on:*

(A) *Mytilus* valves (i) with, or (ii) without, bases of barnacles, in dishes which contained:

		Totals
(*a*) Many barnacles, enclosed in bags of bolting silk, and either		
(i) valves with bases:	8, 6, 4, 7, 10, 6, 5, 4, 7, 8	65
or (ii) valves without bases:	0, 0, 0, 2, 0, 0, 0, 0, 1, 0	3
(*b*) No barnacles and valves without bases:	0, 0, 1, 0, 0, 0, 0, 1, 0, 0	2

(B) Similar pieces of slate which bore:

		Totals
(*a*) Bases of barnacles which were		
(i) Untreated:	12, 11, 11, 8, 12, 12, 10, 6	82
(ii) Incompletely covered with celloidin:	0, 0, 3, 3, 1, 2, 0, 0	9
(iii) Completely covered with celloidin:	0, 0, 0, 0, 0, 0, 0, 0	0
(*b*) No bases	0, 0, 0, 0, 0, 0, 0, 0	0

In further experiments, setting was prevented by covering barnacle bases with a thin but continuous film of nitrocellulose, by painting with 2% celloidin in ether-alcohol. After drying under reduced pressure setting occasionally followed, but subsequent examination showed that the film was cracked in many places, and that the cyprids had settled in the cracks and on the celloidin adjoining them. When the film was hardened by immersion in 90% alcohol, and then washed in hot water to remove the ether-alcohol, it was entirely effective in preventing setting, though such a film would be permeable to most water-soluble substances. It may be inferred that cyprids do not settle readily, unless they have come into physical contact with settled barnacles or their bases.

8. PROPERTIES OF ACTIVE SUBSTANCE

The reaction of cyprids to bases enabled the following experiments to be carried out, to test the stability and chemical nature of the substance to which they respond. Pieces of slate, covered with the bases of recently detached *B. balanoides*, were treated in various ways and then each piece was kept for 24 hr. in a dish of sea water, containing twelve cyprids. Control slates without bases were treated similarly and exposed to cyprids under similar conditions, whilst parallel experiments were carried out with slates bearing untreated bases. Cyprids settled readily within 24 hr. on all the untreated slates, but on none of the bare control slates (Table 7G). The presence or absence of settled cyprids on the treated slates, after a similar period of exposure, therefore showed whether or not the substance which induces setting had resisted the treatment.

(i) *Stability to high temperatures*

Batches of four slates were exposed for 30 min. to various temperatures in an electric oven, allowed to cool, washed, wiped and presented to cyprids. Setting occurred on all the slates which had been exposed to temperatures up to and including 200° C., on two out of four slates exposed to 250° C., on one out of four slates exposed to 275° C. (when charring of the bases occurred) and on none exposed to 300° C. or to higher temperatures (Table 7A). The active substance seems to have been unaffected by heating to 200° C., but gradually destroyed at about 250° C.

(ii) *Prolonged treatment with solvents*

The experiments recorded in Table 7B showed that the bases remained active after treatment with various solvents, which were afterwards removed by drying under reduced pressure, or by washing overnight in running tap water. Chloroform and toluene were used as fat solvents, whilst an alcoholic solution of phenol and aqueous solutions of urea and sodium sulphide were used because of their strong solvent action on some proteins and substances containing the peptide link.

(iii) *Treatment with acids and alkalis*

Boiling in dilute acids, or treatment with cold concentrated acids, had little or no effect, but no setting occurred after treatment with boiling strong acids (Table 7C). Setting was unaffected by cold caustic alkalis, but reduced after brief boiling in these and prevented by prolonged boiling (Table 7D).

Table 7. *Numbers of* Balanus balanoides, *out of similar batches of twelve cyprids, setting within 24 hr. on slates bearing bases of recently detached barnacles, which had been treated with:*

(A) Heat:	
110° C. for 30 min.	5, 4, 10, 6
155° C. for 30 min.	4, 1, 6, 7
175° C. for 30 min.	2, 8, 11, 5
200° C. for 30 min.	2, 10, 3, 3
250° C. for 30 min.	6, 9, 0, 0
275° C. for 30 min.	1, 0, 0, 0
300° C. for 30 min.	0, 0, 0, 0
320° C. for 30 min.	0, 0, 0, 0
350° C. for 30 min.	0, 0, 0, 0
480° C. for 30 min.	0, 0, 0, 0
(B) Solvents:	
Sea water for 7 days*	5, 2, 6, 6
Running tap water, 10 hr.	7, 7, 9, 10
Chloroform, shaken for 2 hr.	1, 2, 6, 9
Toluene at 100° C., 3 hr.	10, 9, 10, 7, 10, 12, 9, 11
50 % phenol in propyl alcohol, 8 hr.	7, 8, 10, 11
Cold conc. urea, 10 hr.	9, 10, 7, 7
Sodium sulphide, 2 hr.	10, 7, 10, 0†
(C) Acids:	
Warm dilute HCl, 2 hr.	11, 5, 5, 3
Boiling N/5-HCl, 1 hr.	9, 5, 12, 12, 12, 11, 6, 2, 1, 0
Cold 2N HNO_3, 1 hr.	12, 10, 12, 9
Cold conc. HCl, 15 min.	4, 4, 4, 7, 10, 9, 8, 10
Cold conc. HNO_3, 25 min.	4, 1, 5, 0
Cold conc. H_2SO_4, 20 min.	6, 3, 4, 3
Hot conc. H_2SO_4, 10 min.	0, 0, 0, 0
Boiling 4N HCl, 2 hr.	0, 0, 0, 0
Boiling 2N HCl, 1 hr.	0, 0, 0, 0, 0, 0, 0, 0, 2, 0
Boiling 2N HNO_3, 1 hr.	2, 0, 0, 0
(D) Alkalis:	
Cold 2N NaOH, 1 hr.	3, 7, 10, 6
Boiling 4N NaOH, 3 min.	5, 5, 0, 0
Boiling 4N KOH, 3 min.	3, 0, 2, 2
Boiling 4N NaOH, 2 hr.	0, 0, 0, 0
Boiling N NaOH, 1 hr.	0, 0, 0, 0, 0, 0, 0, 0, 0, 0
(E) Reagents which attack proteins:	
Pepsin and warm dil. HCl, 6 hr.	6, 5, 12, 9, 2, 6, 2, 8
40 % formaldehyde, 1 hr.	5, 12, 9, 10, 9, 4, 11, 6
10 % formaldehyde, 6 years*	6, 9, 7, 6, 5, 4, 6, 8
10 % benzoquinone in alcohol, 1 hr.	7, 4, 1, 11, 4, 5, 6, 8
Ice-cold diazonium solution, 30 min.,	10, 7, 7, 5
Mercuric chloride, 1 hr.	0, 0, 0, 0
(F) Oxidizing agents:	
Hydrogen peroxide, 6 hr.	8, 6, 4, 6
Sodium hypochlorite, 1 hr.	0, 0, 0, 0
(G) Controls:	
Slates with untreated bases	8, 11, 11, 9, 7, 6, 9, 12 10, 12, 10, 6, 12, 8, 12, 5
Slates without bases	0, 0, 0, 0, 0, 0, 0, 0 0, 0, 0, 0, 0, 0, 0, 0

* *Mytilus* valves used in these experiments.

† Dead algae on surface may have been responsible for this negative result.

(iv) *Treatment with reagents which react with proteins*

Cold concentrated nitric acid turned the bases yellow (xanthoproteic reaction) but did not altogether prevent subsequent setting (Table 7C). The experiments recorded in Table 7E showed that setting readily followed treatment with the following reagents: pepsin, in warm acid solution (which would have attacked accessible peptide links); formaldehyde (which would have reacted with amino groups); benzoquinone (tanning agent); and freshly prepared, ice-cold diazonium solution (which would have reacted with phenolic side chains). After immersion in a solution of mercuric chloride (used on account of the strong adsorption of mercuric ions by carboxyl groups) slates were unfavourable for setting, in spite of very prolonged washing, but the cyprids became sluggish and may well have been affected by de-adsorbed mercury.

(v) *Treatment with oxidizing agents*

Setting occurred readily after treatment with hydrogen peroxide, but not after treatment with sodium hypochlorite, the corrosive agent which dissolves quinone-tanned proteins (Table 7F). This was observed under the binocular microscope to dissolve the bases.

A few similar experiments with bases and cyprids of *Elminius modestus* indicated that these cyprids, like those of *Balanus balanoides*, respond to a substance which is attacked by boiling alkali or cold hypochlorite, and is more resistant to acid than to alkali. The results given in the last column of Table 3 show that the activity of the substance is not affected by prolonged treatment with formaldehyde.

9. PROBABLE NATURE OF ACTIVE SUBSTANCE

These chemical tests throw considerable light upon the nature of the substance to which the cyprids respond. Clearly it is not readily soluble in aqueous or alcoholic media, or in fat solvents, while the heating experiments show that it cannot be in the least volatile. These properties rule out any simple organic substance, and suggest that either a large molecule or a natural polymer is involved. This is in keeping with the specific character of the gregarious effect, which implies small variations in structure between species, such as might be expected in polymeric substances. The resistance of the substance to all but the most corrosive agents further reinforces this view. Its resistance to sodium sulphide rules out a keratin type of protein, while its greater sensitivity to dilute alkalis than to dilute acids rules out chitin. Quinone-tanned proteins, on the other hand, are known to be attacked by hypochlorites, as is found here. Microchemical tests (Knight-Jones & Crisp, 1953) have indicated that the epicuticle of barnacles, like that of other arthropods (Pryor, 1939*a*, *b*; Dennell, 1947), contains quinone-tanned proteins, whilst the cement in the bases is probably of similar material (Thomas, 1944). Darwin (1851–54) remarked that this cement resembles chitin, but is more resistant to acids and less resistant to strong alkalis, and Pyefinch & Downing (1949) suggested that it is a quinone-tanned protein. It seems highly probable that the gregarious response is due to contact with quinone-tanned proteins forming the epicuticle of settled individuals. Such materials occur in many phyla (Brown, 1950), so their exact composition is likely to vary widely and may well be subject to specific differences.

It is difficult, however, to suggest a chemical basis for recognition of such substances, or to analyse the response further. It seems safe to assume that the response is not to phenolic groups, as these would be coupled and rendered insoluble by diazotization, nor to amino groups, since formaldehyde has no effect. The ineffectiveness of strong proteolytic enzymes, of nitration and of extremes of heat which would normally affect proteins profoundly may well be due to a protein framework having been stabilized and protected by a natural tanning process, but its resulting inertness would be an obstacle to recognition by any chemo-sensory mechanism. Perhaps the antennular suckers secrete an enzyme capable of attacking it, for such an enzyme would help cyprids of parasitic cirripedes to penetrate the host's cuticle. It is possible that barnacle cyprids respond to some system of greater than molecular dimensions, such as a pattern on the cuticular surface, but microscopical examination revealed no pattern which was common to both the bases and the parietal plates of adult barnacles.

10. ECOLOGICAL SIGNIFICANCE OF GREGARIOUSNESS DURING SETTING

Field experiments with *Elminius modestus* showed that similar bare surfaces were colonized much more rapidly on shores where adults were abundant, than on shores where there were no barnacles (Knight-Jones & Stevenson, 1950). The behaviour revealed by the experiments of Table 4 explains this effect (p. 589). Many of the cyprids, which settled on the experimental surfaces in the field, would have been stimulated by previous encounters with barnacles on other surfaces nearby, which were too crowded or too muddy to be favourable for setting.

A field experiment with *Balanus balanoides* was carried out at Port Dinorwic, by the Menai Straits, where a beach of slates had been formed by dumping unwanted material from a quarry wharf. Early in 1952, and for some months previously, dumping had been concentrated at one place, and the slates constituting this new area of beach bore no adult barnacles, for they had not been exposed during the setting season of the previous year. Elsewhere barnacles were common on slates, most of which had lain on the shore for many years. In May 1952 setting of *B. balanoides* occurred quite heavily on slates everywhere along this shore except on the new beach where there were no adults; this strongly recalled remarks by Moore (1939) on the slowness with which a new rocky shore at Plymouth became colonized by barnacles.

Eight slates covered with the bases of recently detached barnacles were prepared for the experiment, and four were exposed on the new beach, where no settlement was taking place, whilst the other four were exposed under similar conditions about 30 m. away, where barnacles were setting abundantly. Eight slates which had never borne barnacles were exposed under similar conditions alongside them. After 5 days the slates were examined and the barnacles which had settled on similar areas of each were counted (Table 8A). Tardy colonization of the new beach had begun, for setting was becoming heavier everywhere. An analysis of variance (Table 8B) was applied to the results, which were first subjected to a square root transformation as this seemed the most appropriate in fulfilling the requirements discussed by Bartlett (1947) and Barnes (1952). The analysis indicated that the differences between slates which bore bases and those which bore none are highly significant ($P < 0{\cdot}001$). The considerable numbers of cyprids setting on those slates bearing bases, which were exposed on the new beach, show that there was nothing unfavourable for setting there except the absence of settled barnacles. The differences between the eight slates exposed on the old established part of the beach, and those exposed on the new part, are barely significant ($P < 0{\cdot}05$). Some difference would be expected, for many of the cyprids visiting surfaces on the old beach would have been previously stimulated by contact with barnacles on neighbouring surfaces. There was negligible interaction between the two main effects, and the interaction term was therefore included in the error variance.

Table 8

(A) Numbers of *Balanus balanoides* setting in three days on similar areas 12 × 2 cm. of sixteen slates which either:

	(*a*) Bore bases of numerous barnacles,	or (*b*) had not previously borne barnacles
and which were exposed in batches of four on:		
(i) an old-established beach where barnacles were abundant	$\underbrace{105,\ 83,\ 115,\ 98}_{401}$	$\underbrace{30,\ 16,\ 2,\ 0}_{48}$
(ii) an adjoining new beach where barnacles were absent	$\underbrace{91,\ 25,\ 64,\ 55}_{235}$	$\underbrace{0,\ 10,\ 0,\ 4}_{14}$

(B) Analysis of variance applied to square root transformations of the results given above.

Source of variation	Sum of squares	Degrees of freedom	Mean square	Variance ratio
Presence or absence of bases on slates	181·5	1	181·5	57·4
Exposure on old or new beach	15·5	1	15·5	4·91
Interaction	1·2	1	1·2	—
Replicates (error)	37·9	12	3·16	—
Total	236·1	15	—	—

Field observations for some years in Essex, Cornwall and North Wales have indicated that comparatively few larvae settle outside the zone occupied by the adults, except at times of unusually heavy settlement. At such times, when cyprids are very numerous, even isolated bare surfaces collect abnormally undiscriminating pioneer settlers, which are soon followed by gregarious individuals.

The level of discrimination shown during setting seems to be subject to individual variation, and is probably lowered slightly after prolongation of the planktonic life, as in *Spirorbis* larvae (Knight-Jones, 1953). The normally gregarious reaction must aid breeding, since most species of barnacles appear to be cross-fertilizing (Crisp, 1950), and must also be of value in leading cyprids to habitats which have proved suitable for the survival of the species. This is particularly necessary in *Balanus balanoides*, which thrives only in the tidal zone.

SUMMARY

Laboratory experiments showed that cyprids of *Balanus balanoides* settled readily only when offered surfaces which bore settled barnacles of their own species, or the cemented bases left on surfaces from which these barnacles had been removed. Without such surfaces they proved to be capable of postponing setting for at least 2 weeks, and they chose such surfaces in preference to similar surfaces which bore related species. Cyprids of *B. crenatus* and *Elminius modestus* were also capable of making this choice.

Setting usually occurred in the immediate vicinity of barnacles or bases, but some cyprids crawled short distances away during their final reconnaissances. When they encountered barnacles attached to glass, they were stimulated to proceed with setting, but they swam away from this unfavourably smooth surface and settled on bare stones nearby.

Fragments from various parts of the body, placed upon surfaces which had never borne barnacles, made these somewhat more favourable for setting. Since attached bases alone produced a strong reaction, the cyprids probably respond to contact between their groping antennules and some substance in the cuticle. They did not settle readily when prevented from making contact with adults by a barrier of bolting silk, or with bases by a thin film of nitrocellulose; chemosensory perception of a water-soluble substance, emanating from settled barnacles, is therefore unlikely to be involved.

The power of the bases to induce setting was retained after heating to over 200° C., but not after heating to 275° C., when charring occurred. It withstood prolonged washing in water, heating with fat solvents and dilute acids, and treatment in the cold with caustic alkalis, concentrated acids, pepsin, formaldehyde, benzoquinone, sodium sulphide, phenol, urea, and diazonium solution. It was destroyed in the cold by sodium hypochlorite, which was observed to dissolve the bases, and also by hot concentrated acids and alkalis. The chemical properties of the active substance are thus entirely consistent with those of quinone-tanned proteins, which are known to form the epicuticle and attachment cement.

A field experiment showed that similar behaviour occurs under natural conditions. It leads cyprids to suitable habitats and it facilitates breeding.

I am indebted to Dr D. J. Crisp for most valuable advice and criticism throughout all stages of this work. As our Station was not ready for occupation, Prof. F. W. Rogers Brambell, F.R.S., and Prof. Stanley Peat, F.R.S., kindly provided facilities in the Departments of Zoology and Chemistry at Bangor. Further experiments were carried out at the Fisheries Laboratory, Burnham-on-Crouch, by kind permission of Mr R. E. Savage. I am also grateful to Dr P. N. J. Chipperfield and Miss Emily Clay, of the I.C.I. Marine Paints Laboratory, Brixham, for providing material.

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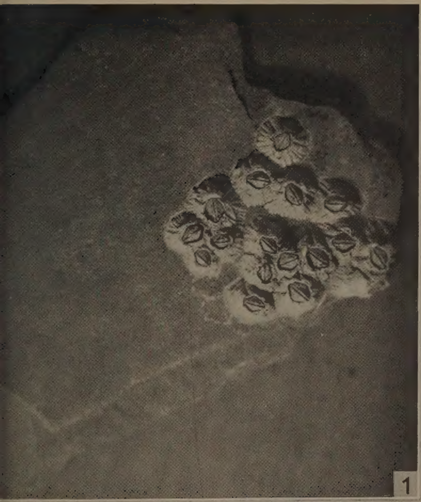
EXPLANATION OF PLATE 16

Fig. 1. Slate bearing a patch of adult *Balanus balanoides*, the position of which has been outlined by a deeply scratched groove. This is seen more clearly in fig. 2.

Fig. 2. The slate shown in fig. 1, after the adult barnacles had been removed, leaving behind their almost invisible membranous bases. Cyprids of *B. balanoides* have been allowed access to the whole slate, but have settled almost exclusively on and amongst the bases of the adults.

Fig. 3. Slate bearing a mixed population of *B. crenatus* (smooth margins) and *B. balanoides* (indented margins).

Fig. 4. The slate shown in fig. 3, after the adult barnacles had been removed and cyprids of *B. balanoides* had been allowed access to it. The calcareous bases of *B. crenatus* are easily visible and the relative positions of the membranous bases of *B. balanoides* can be determined from fig. 3. Few cyprids have settled amongst the bases of *B. crenatus*, but many have settled on or near the bases of their own species and many have settled on an uneven area, in the right-hand bottom corner of the photograph, which was not occupied by barnacles when the slate was taken from the shore. In this area settlement and orientation in grooves (low thigmo-kinesis) are well illustrated.



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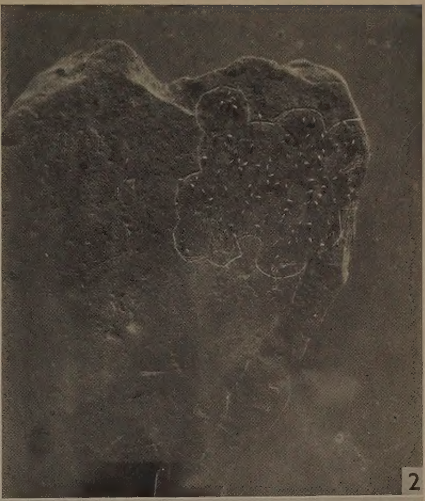

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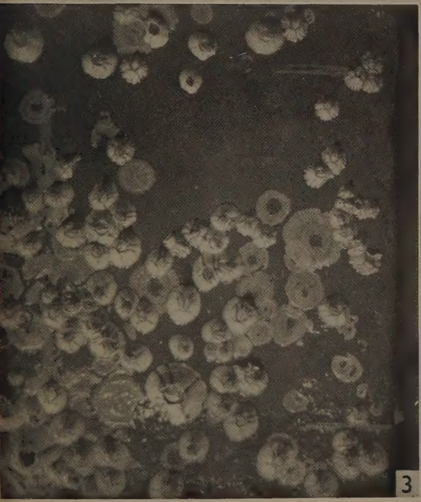

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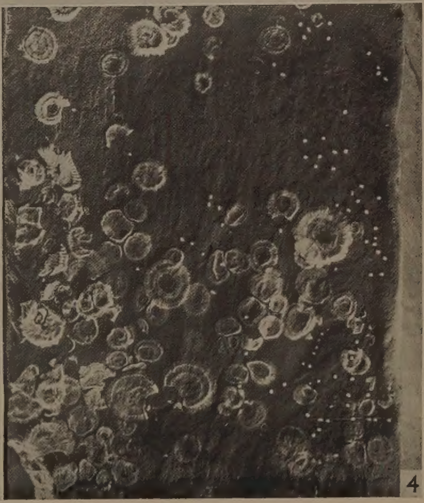

4

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